THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

(Ph.D.)

INFLUENCE OF THE ADENOSINE/A_{2A} RECEPTOR AND TISSUE TRANSGLUTAMINASE ON THE INFLAMMATORY RESPONSE OF MACROPHAGES

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LIST OF ABBREVIATIONS

AC/APO:	Apoptotic Cell
ACAMP:	Apoptotic-Cell-Associated Molecular Pattern
ADA:	Adenosine Deaminase
ADO:	Adenosine
AP-1/4:	Activator Protein 1/4
AR:	Adenosine Receptor
ArgI/II:	Arginase I/II
ATF:	Activating Transcription Factor
C/EBP:	Ccaat-Enhancer-Binding Protein
cAMP:	Cyclic Adenosine Monophosphate
CCR:	C-C Chemokine Receptor
CD:	Cluster of Differentiation
CREB:	cAMP Response Element-Binding Protein
CXCL:	C-X-C Motif Chemokine Ligand
ECM:	Extracellular Matrix
eiF4E:	Eukaryotic Translation Initiation Factor 4E
ERK:	Extracellular Signal-Regulated Kinase
FAK:	Focal Adhesion Kinase
HMGB-1:	High-Mobility Group protein B1
ICAM:	Inter-Cellular Adhesion Molecule
IFNγ:	Interferon γ
IL:	Interleukin
IL-1ra:	IL-1 Receptor Antagonist
IP3:	Inositol Trisphosphate
IP-10	interferon-gamma inducible protein 10 kD
ITAM:	Immunoreceptor tyrosine-based Activation Motif
ITIM:	Immunoreceptor tyrosine-based Inhibitory Motif
IκBα:	NF-κB Inhibitor α
JNK:	c-jun Kinase 1
KC:	cytokine-induced neutrophil-attracting chemokine (CXCL1)
LDL:	Low-Density Lipoprotein
LPS:	Lipopolysaccharide
LXR:	Liver X Receptor
MAPK:	Mitogen-Activated Protein Kinase
MEK:	MAP Kinase Kinase (MKK)
MFG-E8:	Milk Fat Globule-EGF Factor 8 protein
MHC:	Major Histocompatibility Complex
MIP-2:	Macrophage Inflammatory Protein 2 (CXCL-2)
mTOR:	Mammalian Target of Rapamycin
NCoR:	Nuclear Receptor Co-Repressor
NF 1:	Nuclear Factor 1
NF-κB:	Nuclear Factor κ-light-chain-enhancer of Activated B cells
NO:	Nitric Oxide
NOS:	Nitric Oxide Synthase
PAF:	Platelet-Activating Factor
PAMP:	Pathogen-Associated Molecular Pattern
PGE2:	Prostaglandin E2
PI3K:	Phosphatidylinositol 3-Kinases
PKA/PKC:	Protein Kinase A/C

PLC:	Phospholipase C
PPAR:	Peroxisome Proliferator-Activated Receptor
PRR:	Pattern Recognition Receptor
PS:	Phosphatidylserine
PSR:	Phosphatidylserine Receptor
RTK:	Receptor Tyrosine Kinase
S1P:	Sphingosine 1-Phosphate
SFK:	Src Tyrosine Kinase
SIRP-α:	Signal Regulatory Protein α
SOCS:	Suppressor of Cytokine Signaling
STAT:	Signal Transducer and Activator of Transcription
TG2:	Tissue Transglutaminase
TGF-β:	Transforming Growth Factor β
TLR:	Toll-like Receptor
TNF-α:	Tumor Necrosis Factor-α
TSP:	Thrombospondin

1. INTRODUCTION

1.1. MACROPHAGES: SENTINELS OF THE INNATE IMMUNE SYSTEM

Macrophages are large, resident phagocytic cells in tissues, which originally identified by Aschoff in 1924 (Aschoff 1924; Geissmann et al., 2010). They are found ubiquitously in the body.

1.1.1. The functions of macrophages

Macrophages have a role in orchestrating and executing most homeostatic, immunological, and inflammatory processes including recognition, phagocytosis and clearance of invading pathogens through the expression of pattern recognition receptors (PRRs) and the up-regulation of cytotoxic molecules; immune modulation through the production of cytokines and chemokines, antigen presentation, regulation of T cell activation and differentiation; resolution of inflammation, promotion of healing through induction of matrix synthesis, fibroblast proliferation, angiogenesis, and the clearance of cellular debris (Zhang and Mosser, 2008; Correll *et al.*, 2004, Haskó *et al.*, 2007).

1.1.2. Polarization of macrophages

Macrophages undergo activation in response to a broad spectrum of environmental signals. The type, timing, and concentration of these stimuli determine the range of immune responses (Cassol *et al.*, 20010). Polarized macrophages have been broadly classified into two groups: M1 and M2 macrophages. These groups differ in terms of receptors, cytokine and chemokine expression, and effector functions (*Fig.1*).

- Classically activated (M1) or **inflammatory macrophages** are primed by IFNγ, TNF-α or LPS. They can be identified by a variety of biochemical and functional criteria *(Fig.1)*, including the enhanced ability to present antigen and kill intracellular pathogens (Mosser 2003). In addition, M1 macrophages exhibit a Th1-like phenotype, promoting inflammation, ECM destruction, and apoptosis (Benoit *et al.*, 2008, Chawla, 2010).

- Alternatively, activated (M2) macrophages are immunomodulators and are poorly microbicidal (Benoit *et al.*, 2008). M2 macrophages include at least three subsets: M2a, M2b and M2c (Cassol *et al.*, 2009). **Phagocytosis of apoptotic cells renders macrophages to M2c deactivated phenotype**, in which cells are refractory to the classical stimulus LPS, and enhance the release of deactivating cytokine IL-10 and TGF-β (Cassol *et al.*, 2009).

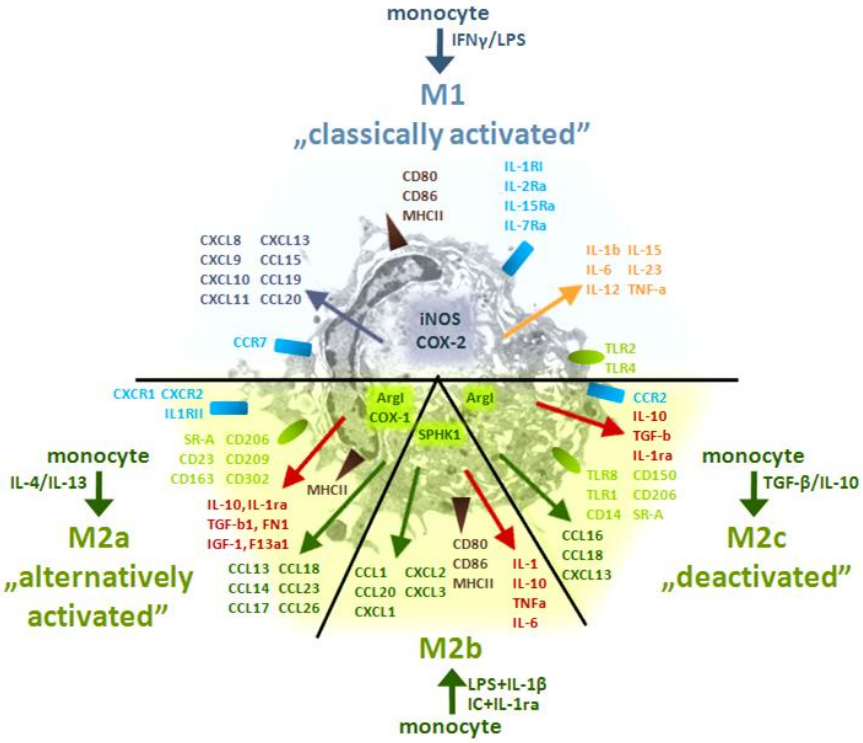


Figure 1. Properties of polarized macrophages. *(adapted from Benoit et al., 2008).*

Thus, macrophage activation can be either pro-inflammatory or anti-inflammatory. Recently, activation has been shown to be plastic, rapid, and reversible, suggesting that macrophage populations are dynamic and may first take part in inflammation and then participate in its resolution (Mantovani *et al.*, 2004).

Although activation is critical for the effective immune response, inappropriate and sustained activation/polarization of macrophages can lead to tissue damage, immune dysfunction, and disease pathology (e.g. autoimmune diseases such as rheumatoid arthritis, SLE and multiple sclerosis; Behrens 2008).

1.2. APOPTOTIC CELL CLEARANCE

Cells die at different stages of life as part of embryonic development and normal tissue turnover, thus the term programmed cell death, or apoptosis, where death is expected and part of the well-being of healthy tissues. Moreover, efficient removal of apoptotic cells not only eliminates abnormal, non-functional or harmful cells, but also helps sculpt organs and maintain the homeostasis of the host (Zhou and Yu 2008).

1.2.1. Apoptosis

The term apoptosis was proposed originally by Kerr, Wyllie and Currie in 1972. They observed characteristic microscopic structural changes in cells undergoing this form of programmed cell death (Kerr *et al.,* 1972). These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. In addition, the plasma membrane of the apoptotic cell (AC) is radically different from its viable counterpart: it is fundamentally altered in architecture, especially with respect to lipid topology, with loss of phospholipid asymmetry and, in particular, oxidation and redistribution of the anionic phospholipid PS from the inner plasma membrane leaflet to the outer (Gregory and Pound 2010).

Recent studies have suggested that the apoptotic program is rather closely linked to the prompt removal of these cells (Ravichandran 2010). Unlike necrosis, apoptosis produces cell fragments called apoptotic bodies that phagocytic cells are able to engulf and quickly remove before the contents of the cell can spill out onto surrounding cells and cause damage (Alberts 2008). The engulfment of ACs is performed by both professional phagocytes (such as macrophages and dendritic cells) and by nonprofessional "neighbouring" phagocytes (such as epithelial cells, endothelial cells, and fibroblasts) (Elliott and Ravichandran 2010). Under normal circumstances, phagocytosis of ACs is fast and effective without causing inflammation and immune response; instead, ACs have an immunosuppressive effect (Kruse *et al.,* 2010). Thus beyond its role in tissue homeostasis, AC clearance is essential to avoid inflammation.

1.2.2. Removal of apoptotic cells

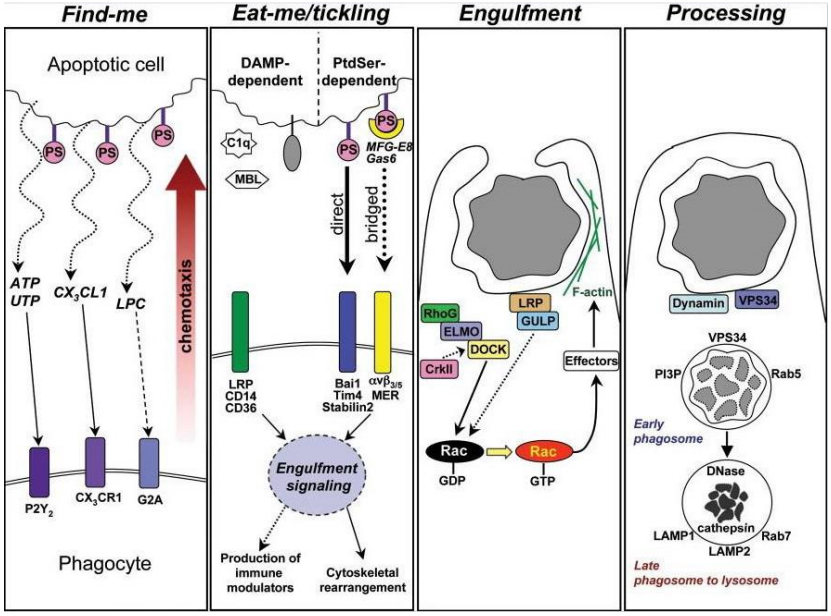


Figure 2. Steps of apoptotic cell clearance.

(Elliott and Ravichandran 2010).

In the past decade, several broadly defined steps have been identified in the recognition and removal of ACs by phagocytes (Fig. 2). Each step appears to be tightly regulated by signaling events to ensure swift and efficient clearance (Elliott and Ravichandran 2010).

1.2.3. Recognition of apoptotic cells

The engulfment of ACs orchestrated through the interaction of numerous recognition or find-me and eat-me signals, bridging molecules, and engulfment receptors (Fadeel *et al.*, 2010).

"Find-me" signals

Phagocytes, which are not necessarily in direct contact with their future meals but rather require initial, directed migratory steps in the recognition process, respond to chemotactic factors released by ACs (Gregory and Pound 2010). Find-me signals may also influence immunogenic versus nonimmunogenic responses to ACs (Ravichandran 2010). Several soluble chemoattractant find-me signals have been recently defined, including triphosphate nucleotides (ATP/UTP), lysophosphatidylchloline (lysoPC), the adhesion molecule and chemokine

CX_3CL1 (fractalkine) and sphingosine 1-phosphate (S1P) (Elliott *et al.,* 2009, Lauber *et al.,* 2003; Truman *et al.,* 2008, Gude *et al.,* 2008). However, whether the chemokine is directly produced by the apoptosing cells or in response to them is not yet clear (Erwing and Henson, 2008).

"Eat-me" signals

Discovery of the involvement of pattern recognition receptors (PRRs) in the clearance of ACs led to the proposal that ACs present structures to phagocytes that have been termed—apoptotic-cell-associated molecular patterns (ACAMPs) because of their purported structural homology with pathogen-associated molecular patterns (PAMPs) (Gregory and Pound 2010). There are several mechanisms to remove ACs.

Phosphatidylserine (PS)

A hallmark of apoptosis-associated membrane alterations is the externalization of PS (Martin *et al.,* 1995). Phospholipid asymmetry is secured by aminophospholipid translocase, which 'flips' PS back to the inner side of the membrane, once it appears at the outer leaflet. During apoptosis, this translocase activity is disrupted, which prevents retraction of PS from the outer cell surface (Bratton *et al.,* 1997). Additionally, PS exposure is enhanced by caspase-3-dependent activation of phospholipid scramblase, an enzyme that promotes an energy-independent, bidirectional scrambling of all classes of phospholipids across the membrane (Frasch *et al.,* 2000). Moreover, PS oxidation (PS-OX) and externalization is a prerequisite for phagocyte recognition (Kagan *et al.,* 2003).

Other eat-me signals

In addition to PS exposure alterations in other phospholipids -such as glycosylation pattern (i.e. ICAM-3 glycosylation) and the sugar composition of the plasma membrane are also changed during apoptosis (Gregory and Pound 2010). These modifications result in the generation of sites resembling oxidised lipoprotein particles, thrombospondin (TSP) binding sites, sites capable to binding lectins, the complement proteins C1q and C3b and collectins (Erwing and Henson, 2008). In addition, the appearance of several proteins (i.e. calreticulin (CRT), annexin 1, the large subunit of the eIF3a, pentraxin PTX3, urokinase receptor (uPAR) as well as DNA or lyso-phosphatidylcholine (lyso-PC) and on the cell surface are also likely contrib-

utes to ACAMP formation (Ogden *et al.,* 2001, Peng *et al.,* 2007; Park *et al.,* 2009; Gregory and Pound, 2010).

"Don't-eat-me" signals

In addition, loss of inhibitory molecules so called 'don't-eat-me' signals- from the surface of viable cells as a consequence of the apoptotic program also promote interactions of ACs with phagocytes (Gregory and Pound, 2010). For example, ACs downregulate CD47 a molecule whose interaction with macrophage SIRP-α prevents phagocytosis of viable cells (Gardai *et al.*, 2005). Similarly, the platelet endothelial cell adhesion molecule (PECAM/CD31) interacting homotypically with CD31 on potential phagocytes facilitate detachment of living cells and thereby potentially prevents engulfment (Brown *et al.,* 2002).

Overall, the appearance of 'eat-me' signals, combined with the loss of 'don't-eat-me' signals, appears to create a molecular topology that solicits appropriate recognition events and responses by phagocytes selectively to non-viable cells.

1.2.4. Interaction between apoptotic cells and macrophages: the "third synapse"

The surface changes of ACs can either interact directly with receptors on the macrophage surface or bind serum proteins (opsonins) that serve as links between the phagocyte and its apoptotic meal.

Direct recognition

Recognition of PS is mediated directly by integral PS receptors (PSR) of the phagocyte membrane (Gregory and Pound 2010). These recently discovered PSRs are TIM-4 (T cell immunoglobulin- and mucin-domain-containing molecule-4) and the related proteins, TIM-1 and TIM-3 (Park et a., 2009); the seven trans-membrane brain angiogenesis inhibitor 1 (BAI1; Park *et al.,* 2007), and the atypical EGF (epidermal growth factor)-motif containing membrane protein Stabilin-2 (Park *et al.,* 2008, Fadeel *et al.,* 2010, Ravichandran 2010).

Besides the PSRs, numerous other receptors are implicated in AC recognition. Most of these receptors are employed during engulfment of pathogens or recognition of oxidized lipoproteins (oxLDLs). Among them are the scavenger receptors CD36, and its relative SR-BI (Fadok *et al.,* 2001), scavenger receptor A (SR-A) or oxLDL receptor macrosialin CD68, and

lectin-like oxidised LDL particle receptor (LOX-1) (Weigert *et al.,* 2009; Fadok *et al.,* 2001). In addition integrins ($\alpha_v\beta_3$, $\alpha_M\beta_2$, and $\alpha_x\beta_2$), immunoglobulin super-family molecules (e.g. CD31), complement receptors (e.g. CD91/CRT), ATP-binding cassette 7 (ABCA7), and PRRs (e.g. CD14), as well as molecules involved in engagement of sugars (e.g. lectins) also take a part in the recognition process (Gregory and Devitt, 2004; Erwing and Henson, 2008).

Recognition via bridging molecules

While many of the phagocytic receptors bind directly to the ACs, some others require bridgeing molecules for proper recognition. The first bridge molecule identified was thrombospondin.1 (TSP-1) an extracellular matrix glycoprotein, and thought to bind to TSP-1 binding sites on ACs and then bind to the integrin $\alpha_v\beta_3$ and the scavenger receptor CD36 on the phagocyte (Moodley *et al.,* 2003).The plasma factor β2-glycoprotein I binds to its specific receptor upon ligating PS. Receptor tyrosine kinases (RTKs: Axl, Sky, Tyro-3 and Mer) recognize PS via growth arrest-specific 6 (Gas6) and protein S binding, while the milk-fat globule epidermal growth factor 8 (MGF-E8, known as lactadherin) connects PS with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins through its RGD motif (Elliott *et al.,* 2010, Gregory and Pound 2010; Weigert *et al.,* 2009; Kruse *et al.,* 2010; McColl *et al.,* 2009). Other bridgeing molecules are linked to the recognition of altered sugars and/or lipids on the AC surface and include the members of the collectin family surfactant proteins A and D, mannose-binding lectin (MBL) and complements (Vandivier *et al.,* 2002; Kruse *et al.,* 2010). IgM antibodies and acute phase proteins such as C-reactive protein (CRP), have been shown to bind to lyso-PC (Peng *et al.,* 2007).

1.2.5. Engulfment and processing

The direct or indirect ligation of PSRs results in Rac-dependent cytoskeletal reorganization, which ultimately leads to **engulfment** of the AC (Chekeni and Ravichandran, 2011). The activation of those pathways, which are involved in the engulfment of ACs, appear to be regulated by the balance of Rho GTPases, including Rac-1 and RhoA (Vandivier *et al.,* 2006). Rac-1 is induced by the PSRs and CD91 and positively regulates phagocytosis, while RhoA negatively regulates the engulfment through its downstream effector Rho kinase (Vandivier *et al.,* 2006).

The internalized cell corpses are enclosed within membrane-bound vesicles called phagosomes. The resulting AC-containing phagosomes undergo a maturation process involv-

ing fusion steps culminating in the formation of phagolysosomes in which cell corpses are degraded. The regulation of AC **processing** is not well understood, but it is confirmed, that Rho/Rac balance is not only important for AC engulfment but also for phagosome maturation and may have direct consequences for antigen presentation (Erwing and Henson, 2007).

1.2.6. Anti-inflammatory effect of apoptotic cell uptake

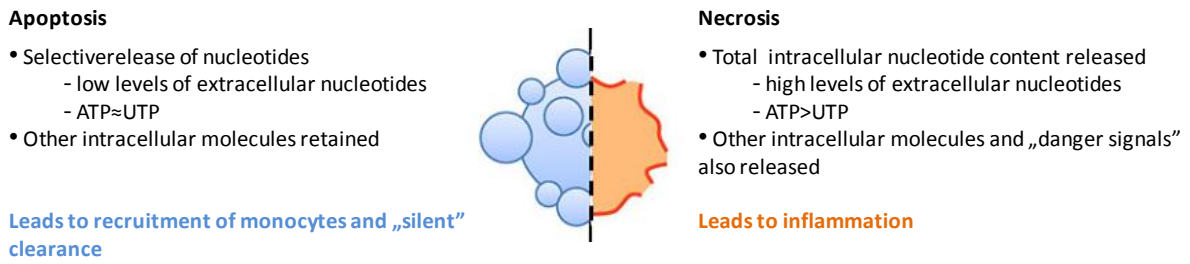


Figure 3. Differential release of molecules from apoptotic and necrotic cells.

(Chekeni and Ravichandran, 2011)

In the late 90s' Voll and co-workers observed that LPS-induced TNF-α and IL-1b expression in monocytes was reduced after co-culture with apoptotic lymphocytes, whereas IL-10 was upregulated (Voll *et al.*, 1997). Since then it was universally accepted that in contrast to PAMP- or necrotic cell-induced inflammatory responses, AC uptake by phagocytes has immunosuppressive effects both *in vitro* and *in vivo* (Torchinsky *et al.*, 2010, Huynh *et al.*, 2002). Moreover, AC uptake actively suppresses TLR-dependent release of pro-inflammatory cytokines, such as IL-6, IL-8, IL-12, IL-1β and TNF-α (Voll *et al.*, 1997). The reduced cytokine expression is primarily associated with gene expression changes and with the inhibition of NF-κB pathway (Cvetanovic and Ucker, 2004, Bonizzi and Karin, 2004).

Direct inhibitory effect of apoptotic cells

The PS-dependent direct suppression of proinflammatory responses is exerted directly upon binding to the macrophage, independent of subsequent engulfment (Cvetanovic and Ucker, 2004). The anti-inflammatory signals can be destinated by PS-containing cell membranes from early apoptotic, late apoptotic, or lysed cells (Fadok *et al.*, 2001). To date, a number of participant signalling elements and pathways have been identified, that mediate this anti-inflammatory effect:

- Activation of MerTK leads to the inhibition of **NF-κB** activation via the **PI3K/AKT** signaling pathway (Sen *et al.*, 2007)
- Ligation of integrin $\alpha_v\beta_3$ triggers different signaling pathways, including MAPKs, PKA and PKC leads to the activation of cyclooxygenase-2 (**COX-2**), and in this way to PGE2 release (Mayoral *et al.,* 2005). COX-2 expression in macrophages by ACs is also facilitated by RNA-binding protein HuR-mediated RNA stabilization (Johann *et al.,* 2008).
- **ITAM or ITIM containing receptors** such as SIRPα and CD31 are also a potencial participant in the immunosuppressive effect of ACs (Gregory and Devitt, 2004). A well characterized example of these competing influences is provided by the FcγRIII which contains ITAM motif and activates macrophages, including promoting phagocytosis and release of pro-inflammatory cytokines. In contrast, the ITIM-containing FcγRIIB inhibits the ITAM-triggered activation (Kluth *et al.,* 2004).
- AC recognition induces **SOCS1 and SOCS 3** (suppressors of cytokine signaling) proteins and suppresses the **Jak-STAT** signalling (Tassiulas *et al.,* 2007).
- Kim *et al.,* identified a novel zinc-finger transcription factor called GC-binding protein (**GC-BP**). GC-BP becomes tyrosine dephosphorylated in the course of AC treatment and binds to the promoter of IL-12 p35 gene, leading to suppression of it's transcription (Kim *et al.,* 2004).
- **cAMP** signaling is considered a hallmark of regulatory macrophages in the resolution phase of inflammation, generally accompanied by COX-2 expression and secretion of high levels of IL-10 (Bystrom *et al.,* 2008).

Phagocytosis of ACs brings in large amounts of cellular lipids into the phagocytes. Recognition of oxidized fatty acids and cholesterol by lipid-sensing nuclear receptors (PPARs and LXRs), is also involved in the clearance of dying cells (A-Gonzalez *et al.,* 2009, Mukundan *et al.,* 2009).

- It is confirmed that both **PPARγ, and PPARδ** play a role in the regulation of both the engulfment and the anti-inflammatory response (Májai *et al.,* 2007, Mukundan *et al.,* 2009). Ligand binding mediates covalent conjugation of SUMO1 (small ubiquitin-like

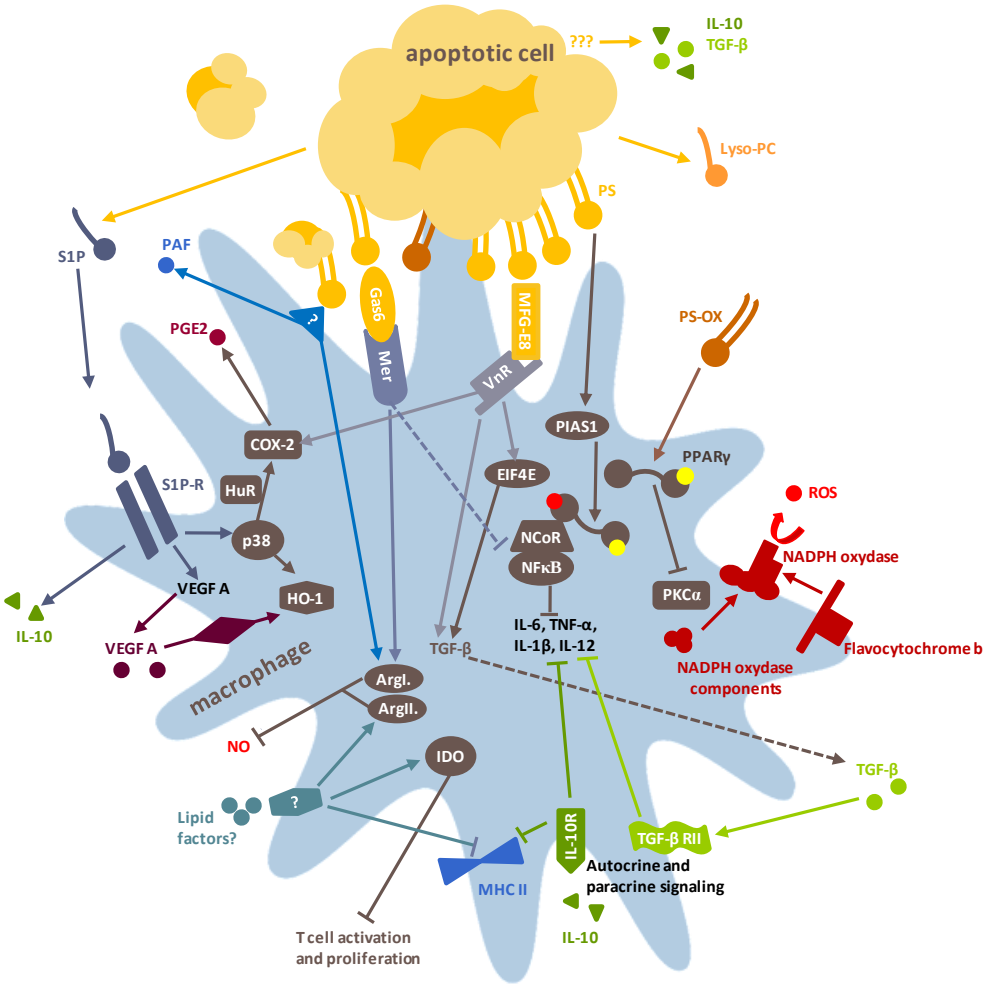


Figure 4. Signaling of ACs towards alternative macrophage activation.

(adapted from Weigert et al., 2009)

modifier) to PPARγ allowing it to bind to NCoR and interfere with clearance of the corepressor complex by blocking its degradation (Straus and Glass, 2007). This maintains the promoter in the repressed state and results in suppression of inflammation. When ligand binds to PPARδ, on the contrary, the transcriptional repressor BCL-6 is released, which then represses the inflammatory-response (Torchinsky *et al.*, 2010).

- Similar to PPARγ, **LXR** upon ligand binding also interferes with clearance of NCoR containing corepressor complexes from the promoters of distinct but overlapping inflammatory-response genes (Straus and Glass, 2007). This regulatory role of LXR is required for the induction of TGF-β and IL-1. In addition LXR induces the expression of MerTK, hereby enhances the clearance of ACs (A-Gonzalez *et al.*, 2009). Similarly, PPARδ promotes the production of C1qb (Mukundan *et al.*, 2009).

Soluble mediator-mediated inhibitory effect of apoptotic cells

In parallel to direct inhibitory effect, the uptake of ACs by phagocytes is thought to suppress autoimmune responses through the PS-dependent release of soluble anti-inflammatory molecules (e.g. IL-10, TGF-β, PAF, PGE2, and S1P) (Voll *et al.,* 1997, Fadok *et al.,* 1998, Weigert *et al.,* 2006). These mediators act in an autocrine/paracrine manner to suppress the production of inflammatory cytokines, chemokines (eg, IL-8 and MIP-2), and lipid mediators (eg, thromboxane A2) (Henson *et al.*, 2001).

IL-10

Interleukin-10 (IL-10) is a pleiotropic cytokine produced by T- and, B-cells and macrophages and possesses both anti-inflammatory and immunosuppressive properties (Moore *et al.,* 1990). Various studies showed that IL-10 is an inhibitor of a broad spectrum of monocyte/macrophage functions, including pro-inflammatory cytokine synthesis, NO production, and expression of MHCII and co-stimulatory molecules such as CD80/CD86 (Moore *et al.,* 2001). In addition, IL-10 emerged as a key regulator suppressing Th1 responses (Kühn *et al.,* 1993).

Zhang et al found that AC derived signals recognized by CD36 induces p38 MAPK phosphorylation and the activation of a yet to be identified protein tyrosine kinase (X). Activation of p38 MAPK leads to the assembly of a Hox complex composed of Pbx-1b, Prep-1, and Meis1 (Zhang *et al.,* 2010). This complex binds to an AC response element (ACRE) in the promoter of IL-10 gene (Chung *et al.,* 2007).

TGF-β

TGF-β is a multifunctional cytokine that regulates numerous physiological processes, including cell growth, differentiation, apoptosis, adhesion, early embryonic development, and extracellular matrix protein synthesis (Chin *et al.,* 1999). TGF-β released by AC-treated macrophages prevents pro-inflammatory cytokine production through inhibition of p38 MAPK and NF-κB pathways. On the other hand, TGF-β just slightly affects the expression of AP-1-regulated genes (Xiao *et al.,* 2002).

During AC clearance, TGF-β mRNA expression is up-regulated by a PS-dependent manner with the participation of p38 MAPK, ERK and JNK (Park *et al.,* 2004). In parallel, the protein translation is also enhanced via a newly defined PSRS/RhoA/PI3K/Akt/mTOR/eiF4E signal-

ing pathway (Xiao *et al.,* 2008). Stabilin-2 is also involved in the activation of signaling pathways leading to TGF-β production (Park *et al.,* 2004).

Sphingosine-1-phosphate (S1P)

S1P described for its anti-inflammatory and anti-apoptotic effects, emerged as a potential candidate for soluble agent during the clearance of ACs (Weigert *et al.,* 2009). Indeed, S1P is released from ACs, reduce TNF-α and IL-12p70 secretion of macrophages, but enhanced the expression of IL-10 and IL-8 (Weigert *et al.,* 2007). Furthermore, AC-derived S1P facilitates the expression of COX-2 (Johann *et al.,* 2008), and heme oxygenase1 (HO-1) in a biphasic manner (Weigert *et al.,* 2009). HO-1 catalyzes the rate-limiting step in the degradation of heme to biliverdin, ferrous iron, and carbon monoxide and is known for its anti-apoptotic and anti-inflammatory properties (Weigert *et al.,* 2009).

Other mediators

An additional mechanism underlying the differential abilities of apoptotic and necrotic cells to stimulate inflammatory responses has been proposed based on the extracellular activity of the DNA-binding protein HMGB-1, which can leak out of necrotic cells triggering inflammatory responses from macrophages, but remains sequestered in ACs (Gregory and Devitt, 2004). Macrophage activation by HMGB-1 is LPS-like, being dependent upon TLR2 and TLR4. Absence of available extracellular HMGB-1 may provide a molecular basis for the lack of pro-inflammatory effects of late/leaky ACs such as lymphocytes (Gregory and Devitt, 2004).

1.3. ADENOSINE

Adenosine (ADO) is an endogenous purine which plays an important role in biochemical processes, such as energy transfer—as adenosine adenosine triphosphate (ATP) and adenosine diphosphate (ADP)—as well as in signal transduction as cAMP. It is also an inhibitory neurotransmitter, and potent anti-inflammatory substance.

1.3.1. Adenosine metabolism

There are several important producer cell types of extracellular ADO. Neutrophils and endothelial cells release large amounts of ADO at sites of metabolic distress, inflammation and infection (Haskó *et al.,* 2007). Activated macrophages (e.g. LPS-treated) can also serve as a major source of extracellular ADO via ATP release and degradation (Ruiz-García *et al.,* 2011). ADO concentrations at its receptors are determined by a variety of processes, which include extracellular and intracellular ADO generation, ADO release from cells, cellular reuptake and metabolism. These processes are closely intertwined and strictly regulated (Haskó *et al.,* 2007).

Intracellular adenosine production

Intracellularly, ADO formation is dependent upon the hydrolysis of AMP by an intracellular 5-nucleotidase or hydrolysis of S-adenosyl-homocysteine (Gessi *et al.,* 2010). The AMP hydrolizing 5'-nucleotidase has two forms (cN-I and cN-II) (Sala-Newby *et al.,* 2000), and they play different roles—cN-I breaking down AMP to ADO and cN-II breaking down IMP and GMP to inosine and guanosine, respectively (Fredholm *et al.,* 2001a).

Extracellular adenosine production

The dephosphorylation of extracellular AMP to ADO is mediated by ecto-5-nucleotidases (e.g. CD39, CD73) that catalyze the breakdown of extracellular adenine nucleotides, such as ATP, to ADO (Zimmermann, 2000). Another potential source of extracellular ADO is cAMP, which is converted by extracellular phosphodiesterases into AMP and thereafter by an ecto-5-nucleotidase to ADO (Fredholm *et al.,* 2001a).

Degradation of adenosine

Adenosine leaving the producer cell is rapidly broken down by adenosine deaminases (ADAs) to inosine, which is also a weak agonist for A_1 receptors in a higher concentration range (>100 μM) or an immunomodulatory molecule for A_{2A} and A_3 receptors (Sipka, 2011). In mammals, there are two isoforms of ADA: ADA1 and ADA2, which are mainly produced by macrophages/monocytes (Conlon and Law, 2004). The primary role of ADA1 is to eliminate intracellular toxic derivatives of adenosine and deoxyadenosine and protect the cells from apoptosis (Zavialov *et al.*, 2010). ADA2 is the predominant form present in human blood plasma and is increased in many diseases, particularly those associated with the immune system: for example rheumatoid arthritis, pulmonary tuberculosis and HIV infection (Zavialov *et al.*, 2010). Overall, decreasing the extracellular amount of ADO ADA enzymes have potential role in the regulation of adenosine receptor-mediated cellular responses.

1.3.2. Adenosine transport

ADO generated intracellularly is transported into the extracellular space mainly via specific bi-directional transporters through facilitated diffusion, though some tissues (e.g., kidney brush-border membranes) express concentrative nucleoside transport protein (CNT1 and CNT2) capable of maintaining high ADO concentrations against a concentration gradient (Fredholm *et al.*, 2001a). Generally, when the intracellular level of ADO is very high, ADO simply diffuses out of cells. Direct release of intracellular adenine nucleotides, such as ATP, that is thereafter converted extracellularly by ecto-ATPase and ecto-ATP-diphosphohydrolase (ecto-apyrase) to AMP and dephosphorylated by ecto-5'-nucleotidase to ADO, can also contribute to the ADO transport (Zimmermann *et al.*, 1998). Extracellularly, ADO concentration is kept in equilibrium by reuptake mechanisms operated through the action of specific transporters. Then inside the cell it is phosphorylated to AMP by ADO kinase or degraded to inosine by ADO deaminase (ADA) (Gessi *et al.*, 2010).

1.3.3. Signaling mechanisms mediating the effect of adenosine – with special regard to A_{2A} receptor subtype

As ADO is unstable and its half-life is limited by deamination or cellular reuptake, increase typically affects only local adenosine receptor (AR) signaling. The cellular response to

ADO strictly depends on the expression of the different AR subtypes, which can be co-expressed by the same cell and serve as active modulators in signal transduction (Gessi *et al.*, 2010). Further factor that determine the net effect of ADO on macrophage function is the coupling efficacy of ARs to intracellular signaling (Haskó *et al.*, 2007).

Adenosine receptors

ARs are expressed densely by all cell types that are involved in orchestrating an inflammatory/immune response, and these cell types include dendritic cells, mast cell, platelets, epithelial cells, and fibroblasts, as well as lymphocytes, NK cells and NKT cells (Haskó and Pacher 2008).

ARs belong to the class A G protein-coupled receptor (GPCR) family. These receptors may also be called purine P1 receptors to distinguish them from the receptors for nucleotides, which belong to either the family P2X or P2Y receptors (Fredholm *et al.*, 1997). Based on their affinity ARs can divide into four subtypes: A1 (A_1R), A2A ($A_{2A}R$), A2B ($A_{2B}R$) and A3 (A_3R) (Haskó *et al.*, 2007; Table 2).

Subtype	Affinity * ($EC_{(50)}$)	Specific agonist	Specific antagonist	G protein	Effect of G protein coupling	High expression	Intermediate expression	Low expression
A_1	**HIGH** (0.31 μM)	CPA CCPA CHA CVT-510	CPX CPT N-0840 WRC-0571	$G_{1/2/3}$	**↓ cAMP** ↑ IP_3/DAG (PLC) ↑ arachidonate (PLA_2) ↓Q, P, N-type Ca^{2+} channels ↑ K^+ channels ↑choline, DAG (PLD) βγ-mediated	Brain (cortex, cerebellum, hippocampus) spinal cord, eye, adrenal gland, atria	Other brain regions, sceletal muscle, liver, kidney, adipose tissue, salivary gland, esophagus, colon, antrum, testis	Lung, pancreas
				G_0				
A_{2A}	**HIGH** (0.7 μM)	CGS21680 DPMA HE-NECA ATL-146E	KW6002 CAFFEINE SCH58261 ZM241,385	G_s	**↑ cAMP**	Spleen, thymus, leukocytes, platelets, olfactory bulb, GABAerg neurons	Heart, lung, blood vessels, peripheral nerves	Other brain regions
				G_{olf}	**↑ cAMP**			
				$G_{15/16}$	↑ IP_3			
A_{2B}	**LOW** (24 μM)	BAY60–6583 LUF5835	XAC MRS1754 ALLOXAZINE MRE2029F20	G_s	**↑ cAMP**	Cecum, colon, bladder	Lung, eye, blood vessels, mast cells, median eminence	Adipose tissue, adrenal gland, brain, kidney, liver, ovary, pituitary gland
				$G_{q/11}$	↑ IP_3/DAG (PLC)			
A_3	**HIGH** (0.29 μM)	IB-MECA Cl-IB-MECA	I-ABOPX MRS1191 MRS1220 MRS1292 MRS1523 VUF5574 MRE3008F20	$G_{i,2,3(o)}$	**↓ cAMP** ↑ IP_3/DAG (PLC) ↑choline, DAG (PLD) ↑ K^+-ATP channels ↑ Cl^- channels βγ-mediated	Testis (rat), mast cells (rat)	Cerebellum, pippocampus, microglia, lung, spleen, pineal	Thyroid, most of brain, adrenal gland, spleen, liver, kidney, heart, intestine, testis
				$G_{q/11}$	↑ IP_3/DAG (PLC)			

Table 1. Adenosine receptors

(Fredholm et al., 2001a, Fredholm et al., 2001b)

The actions of the A_{2A}Rs are complicated by the fact that a variety of functional heteromers composed of a mixture of $A_{2A}R$ subunits with subunits from other unrelated G-protein coupled receptors (e.g. $A_1R/A_{2A}R$, dopamine $D_2/A_{2A}R$, $D_3/A_{2A}R$, cannabinoid $CB_1/A_{2A}R$;

$CB_1/A_{2A}R/D_2$ heterotrimers) have been found, adding a further degree of complexity to the role of ADO activity (Ferré *et al.,* 2008; Fuxe *et al.,* 2005; Torvinen *et al.,* 2005; Ferré *et al.,* 2009; Marcellino *et al.,* 2008). The functional significance and endogenous role of these hybrid receptors is still only starting to be unravelled.

Receptor regulation

Exposure of any GPCR to agonists for shorter or longer times generally leads to the attenuation of the agonist response. ARs are no exception. However, the magnitude of this response and the mechanisms involved seem to vary between the AR subtypes. For example, while A_1R and $A_{2A}R$ appear to desensitize slowly and incompletely, the A_3R desensitizes very rapidly (Fredholm *et al.,* 2001a). In parallel, the gene expression of ARs is highly responsive to alterations in the extracellular environment. In this regard, $A_{2A}R$ expression is exquisitely sensitive to changes in the concentrations of exogenous and endogenous factors involved in inflammation. This is shown by the fact that promoter region of $A_{2A}R$ gene (ADORA2) contains putative regulatory elements such as AP-1, NF 1 and AP-4 elements (Fredholm *et al.,* 2000). Thus, exposure of macrophages to LPS induced a dramatic increase in the expression of ADORA2 mRNA and protein (Haskó and Pacher 2008). In addition, $A_{2A}R$ expression can be stimulated also by protein kinase C or hypoxia also (Fredholm *et al.,* 2001a).

G Protein coupling

The A_1R and A_2Rs were initially subdivided based on their ability to inhibit and stimulate adenylyl cyclase, respectively. Indeed, A_1R and A_2Rs are coupled to G_i and G_s proteins, respectively. The more recently discovered A_3R is also G_i coupled. In addition, there is some evidence that the ARs may signal via other G proteins, and they can couple to more than one G protein (Fredholm *et al.,* 2001a). In most peripheral tissues $A_{2A}R$ coupled to G_s. However, $A_{2A}Rs$ may also couple to pathways involving G-proteins other than G_s (G_{olf} and $G_{\alpha 15/16}$, and $G_{i/o}$) or even to AC-(cAMP)-PKA independent signal transduction pathways (Fresco *et al.,* 2004).

Second messengers and signals

Upon activation of the G proteins, enzymes and ion channels are affected as can be predicted from what is known about G protein signaling *(Table 1).* Thus, A_1R mediate inhibition of adenylyl cyclase, activation of K^+-channels (probably via β,γ-subunits), inactivation of several

types of Ca^{2+} channels, activation of PLCβ, etc. The same appears to be true for A_3Rs. In contrast, both A_{2A}R and A_{2B}Rs stimulate the formation of cAMP, and mobilization of intracellular calcium via IP_3 induction (Fredholm *et al.*, 2001a).

MAPK activation

Several effects of ARs are related to changes in the activity of MAPKs. A_1Rs can activate ERK1/2 via β,γ-subunits of Gi/o G proteins. The A_{2B}R is the only subtype that so far has been shown to activate not only ERK1/2 but also JNK and p38. In HEK 293 cells, the MAPK cascade depends on $G_{q/11}$, PLC, ras, B-raf pathway (Fredholm *et al.*, 2001a).

Activation of A_{2A}Rs also increases MAPK activity via the activation of ERK1/2 using the cAMP-ras-MEK1 pathway (Sexl *et al.*, 1997). However, the signaling pathways used by the A_{2A}R seem to vary with the cellular background and the signaling machinery that the cell possesses. For example, the A_{2A}R-mediated ERK1/2 activation in CHO (Chinese hamster ovary) cells is dependent on G_s-cAMP-PKA-rap1-p68 B-raf-MEK1. On the other hand, the A_{2A}R-mediated activation in HEK 293 cells involves PKC, ras, and sos, but not G_s, cAMP, or PKA, even though cAMP levels do rise in a G_s-dependent manner (Fredholm *et al.*, 2001a). A_{2A}R activation may not only stimulate, but also inhibit ERK phosphorylation. Activation of guinea pig A_{2A}Rs expressed in CHO cells inhibited thrombin-induced ERK1/2 activation. This inhibition was cAMP- and PI3K-dependent. In PC12 pheochromocytomic cell line, the activation of A_{2A}R inhibits nerve growth factor (NGF)-induced ERK1/2 phosphorylation, whereas the activation A_{2A}R alone leads to activation (Fredholm *et al.*, 2001a).

The common feature of all ARs, however, is the positive coupling to ERK1/2 even though the classical cAMP/PKA pathway is both activated (A_2Rs) and inhibited (A_1R/A_3R). Depending on the cellular background, the required signaling elements vary widely, although activation of one of the small GTP-binding proteins p21ras and rap1 is essential (Fredholm *et al.*, 2001a). Via the regulation the commonly used cAMP/PKA patway, which can directly phosphorylate and thereby activate the transcription factor CREB, ARs can directly affect gene expression. This effect is can be carried out by direct binding to gene promoters or indirectly, by competing with NF-κB for an important cofactor, CREB-binding protein (Haskó and Pacher 2008).

1.3.4. Immunemodulatory role of adenosine

It is well recognized that certain naturally occurring purines can exert powerful effects on the immune system. The nucleoside ADO is the best characterized of these purines, as both extracellular and intracellular ADO have been shown to affect almost all aspects of an immune response (Haskó *et al.,* 2000). ADO and its analogues can alter the course of a variety of immune-mediated/inflammatory diseases such as endotoxin shock, rheumatoid arthritis, pleural inflammation, nephritis, or uveitis (Haskó *et al.,* 2000). Based on genetic studies, it is becoming increasingly recognized that A_{2A}Rs represent the major immunoregulatory arm of the ADO-AR, and there is general agreement that A_{2A}Rs serve to down-regulate inflammation and immunity (Haskó and Pacher 2008). In addition, A_{2A}Rs play a key role in the phenotypic switch of macrophages from M1 to M2-like phenotype (Grinberg *et al.,* 2009).

Monocyte/macrophage maturation and proliferation

Evidence indicates that ADO can alter the course of macrophage proliferation and differentiation (Haskó *et al.,* 2007). Furthermore, it is confirmed that M-CSF-induced proliferation of mouse bone marrow macrophages is suppressed by ADO, which is mediated through A_{2B}Rs (Haskó *et al.,* 2007).

Migration of immune cells

Hofer *et al.,* showed that AR engagement affects the migratory activity of dendritic cells (Hofer *et al.,* 2003). In case of neutrophils, A_{2A}R activation leads to decreased transmigration, possibly through inhibition of NF-κB signaling pathways (Säve *et al.,* 2011). In addition, A_{2A}R activation modulates CCR7 expression in both normal and inflammatory environments and in this way takes part in the regulation of macrophage migration to CCR7-specific chemoattractants (Williams and Cronstein, 2011).

Oxidative burst and NO production

The inhibition of polymorphonuclear leukocyte's oxidative burst is predominantly A_{2A}R-mediated (Gessi *et al.,* 2002). However, ADO and its analogs acting via A_3Rs are also potent inhibitors of the respiratory burst of monocyte/macrophages (Thiele *et al.,* 2004). In addition,

$A_{2B}R$ signals decrease oxidase activity and in this way superoxide generation of murine neutrophils (van der Hoeven *et al.*, 2011).

Selective A_1R and $A_{2A}R$ agonists suppress (Hasko *et al.*, 1996), while several AR agonists increase the NO production of LPS-stimulated macrophages (Min *et al.*, 2000). In case of neurons, the A_1R stimulation inhibits NF-κB, thereby reducing the expression of iNOS and NO radicals (Pingle *et al.*, 2007).

TLR-mediated cytokine production

TNF-α

While the inhibitory effect of ADO on the TNF-α production is a well known and wildely accepted phenomenon, the studies are conflicting in the point of the involvement of the receptor subtype and the underlying intracellular mechanism.

- In initial studies the anti-infalmmatory effect of ADO was thought to be mediated mainly by A_2Rs (Bouma *et al.*, 1994), especially by $A_{2B}Rs$ (Feoktistov and Biaggioni, 1997).
- Another early study supports the conclusion that ADO acting via $A_{2A}R$ leads to p38 MAPK-dependent post-transcriptional down-regulation of TNF-α (Kotlyarov *et al.*, 1999).
- Investigations using a combination of knock out animals and pharmacological approaches have confirmed the contribution of $A_{2A}R$, but excluded the involvement of A_3R in the suppression of TNF-α production by LPS-stimulated murine peritoneal macrophages (Hasko *et al.*, 2000). According to Haskó *et al.*, (2000) the $A_{2A}R$-independent portion of the ADO effect appears to be secondary to $A_{2B}Rs$ (Hasko *et al.*, 2000).
- Németh *et al.*, found, that the TNF-α suppressing effect of ADO is mediated by NF-κB-dependent pathway (Nemeth *et al.*, 2003).
- It was also found that the inhibitory effect of A_3R is associated with changes in activation of the AP-1 transcription factor system, whereas it is independent of MAPKs and NF-κB, as well as PKA, PKC, and PLC (Martin *et al.*, 2006).

The reason for the inconsistency between the findings of these studies regarding the role of $A_{2A}R$ is not clear but the differences may be a reflection of the different LPS concentrations and incubation times used (Haskó *et al.*, 2007).

IL-12

Both *in vitro* and *in vivo* studies confirmed the inhibitory effect of ADO on IL-12 production of macrophages (Németh *et al.*, 2007; Scheibner*et al.*, 2009). This effect is dependent, in part, on A_{2A}Rs (Link *et al.*, 2000). As IL-12 has a determining role in the regulation Th1immune response, the IL-12 suppressing effect of ADO seems to be the most important impact by which ADO evolves strong anti-inflammatory effects (Sitkovsky *et al.*, 2004).

- A_{2A}R stimulation decreases the mRNA level of IL-12 p40 mRNA, implying a pre-translational mechanism of action (Link *et al.*, 2000).
- A_3R also negatively regulates IL-12 production (Hasko *et al.*, 1998) via the PI3K/Akt pathway (la Sala *et al.*, 2005).

IL-10

Another anti-inflammatory effect of ADO expresses in the induction of IL-10 production of monocytes/macrophages.

It was found in 2000 that A_{2A}R activation is responsible for the IL-10 induction in human monocytes (Link *et al.*, 2000); probably by the augmentation of nuclear accumulation and DNA binding of C/EBPβ (Csóka *et al.*, 2007; Haskó and Pacher 2008).

1.4. TISSUE TRANSGLUTAMINASE

*Transglutaminases (TGs) are a family of intracellular and extracellular enzymes that catalyze Ca^{2+}-dependent posttranslational modification of proteins. Via this function, TGs participate in many biological processes. Besides its classical protein cross-linking activity, TG2 can function also a G protein (G_h) (*Nakaoka *et al.*, 1994*), protein disulfide isomerase (PDI),a protein kinase or a DNA nuclease (Akar et al., 2007, Park et al., 2010). In addition to its various enzymatic activities, TG2 interacts directly with extracellular molecules, therefore mediating cell-extracellular matrix (ECM) interactions (Park et al., 2010).*

Tissue transglutaminase (TG2, tTG, protein-glutamine-gamma-glutamyltransferase, E.C.2.3.2.13) belongs to the family of transglutaminases catalyze the Ca^{2+}- and thiol-dependent posttranslational modification of proteins by transamidation of available glutamine residues (Szondy *et al.*, 2003). This action results primarily in the formation of ε-(γ-glutamyl)lysine cross-links but includes the incorporation of polyamines into suitable protein substrates as well. The covalent isopeptide crosslink is stable and resistant to proteolysis, thereby increasing the resistance of tissue to chemical, enzymatic, and mechanical disruption (Greenberg *et al.*, 1991).

NAME	SYNONYMS	TISSUE	LOCALIZATION
TG1	TG_K, keratinocyte TG, type 1 TG	epithelia	cytosol, membrane
TG2	TG_C, tissue TG, type 2 TG	ubiquitous	cytosol, nucleus, extracellular
TG3	TG_E, epidermal TG, type 3 TG	epithelia	cytosol
TG4	TG_P, prostate TG, type 4 TG	prostate	extracellular
TG5	TG_X, type 5 TG	epithelia	cytosol
TG6	TG_Y, type 6 TG	unknown	unknown
TG7	TG_Z, type 7 TG	ubiquitous	unknown
FXIII	Factor XIIA, plasma TG, fibrin stabilizing factor	blood plasma, platelets	extracellular
Band 4.2	erythrocyte protein band 4.2	erythrocytes	membrane

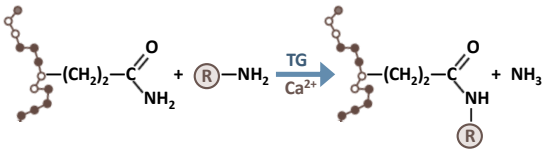


Table 2. Transglutaminases and the reaction catalized by them

TGs catalyze a Ca^{2+}-dependent acyl transfer reaction between the γ-carboxamide group of a peptide-bound Gln residue and the primary amino group (designated R-NH_2) of either a peptide-bound lysine or a polyamine. (Esposito and Caputo 2005)

Eight distinct enzymatically active transglutaminases have so far been described (Greenberg *et al.*, 1991). Among them, TG2 is the only family member expressed in a wide variety of tissues and cell types (Thomazy and Fesus, 1989), and it's abnormal activity is observable

under pathological circumstances and in certain diseases such as Alzheimer's, Huntington and Parkinson's disease and other neurodegenerative disorders (Ruan and Johnson, 2007; Akimov et al; 2000, Iismaa *et al.*, 2009).

1.4.1. TG2: enzymatic activity and function

TG2 is predominantly a cytoplasmic protein, but it is also found in the nucleus and in the mitochondria, on the plasmamembrane and the extracellular cell surface, and in the extracellular matrix (ECM) (Park *et al.*, 2010). The subcellular location of TG2 is critical for regulation of its various biochemical activities, which subsequently trigger diverse downstream events (Park *et al.*, 2010).

Depending upon the substrates, the **cross-linking activity** of TG2 is responsible for different related protein posttranslational modifications such as incorporation of di- and polyamines into proteins, protein–protein cross-linking and site-specific deamidation (Mastroberardino and Piacentini, 2010). By these opposite activities, TG2 plays a similar role in the signaling processes to protein kinases and protein phosphatases. TG2 TGase activity is usually suppressed in cytoplasmic environments due to low intracellular calcium concentrations and high GTP concentrations (Park *et al.*, 2010), but under certain circumstances, TG2 can also act extracellularly: both in membrane-bound, and in released form. In the extracellular space, TG2 has been shown to mediate the interaction between integrins, fibronectin and the extracellular matrix (Mastroberardino and Piacentini, 2010). By this way TG2 modulates cell motility, proliferation, tissue repair (Scarpellini *et al.*, 2009), and inflammation through the regulation of availability of cytokines (i.e.TGF-β) in the ECM (Nunes *et al.*, 1995). In addition, TG2 translocated to the nucleus can cross-link histones (Mishra *et al.*, 2006) which suggest a novel role for TG2 in the regulation of chromatin structure. Moreover it can cross-link transcription factors such as Sp1 (Tatsukawa and Kojima 2010), and trigger gene expression changes (Balajthy *et al.*, 2006, Filiano *et al.*, 2010).

When associated with the plasma membrane, TG2 acts as a **G-protein ($G_{\alpha h}$)** (Nakaoka *et al.*, 1994) in the presence of low calcium levels, binding and hydrolysing GTP to GDP with an affinity and a catalytic rate similar to the alpha subunit of large heterotrimeric G-proteins and small Ras-type G-proteins (Mastroberardino and Piacentini, 2010). By this function TG2 involved in signalling from $\alpha_{1B/D}$ adrenergic receptors to downstream effectors such as phospholipase Cδ1 (Collighan and Griffin, 2009).

Finally, a Ca^{2+}-independent **protein disulphide isomerase (PDI) and kinase activity** has been proposed for this enzyme (Mishra *et al.*, 2006). While the PDI activity catalyses the formation of disulphide bonds in mitochondrial components (e.g. complex I, IV and V; ANT1) (Mastroberardino *et al.*, 2006; Malorni *et al.*, 2009); the kinase activity allows the phosphorylation of histone proteins (Mishra *et al.*, 2006), retinoblastoma protein (Rb) (Mishra *et al.*, 2007), p53 (Mishra and Murphy, 2006) and insulin-like growth factor-binding protein-3 (IGFBP-3) (Mishra and Murphy, 2004). The posttranslational modification of these substrates plays an important role in the energy production, apoptosis, chromatin structure and function.

1.4.2. The consequences of TG2 deficiency –with special regard to the clearance of apoptotic cells and inflammation

TG2 deficiency fundamentally impairs the capacity of macrophages to ingest apoptotic cells and promotes dysregulated macrophage release of TGF-β and other cytokines (i.e. IL-12), dyregulated expression of ABCA1, and defective autoinhibition of inflammation modulated by apoptotic cell uptake *in vivo* (Boisvert *et al.*, 2006). Although $TG2^{-/-}$ mice are developmentally normal, they develop splenomegaly and immune complex glomerulonephritis beyond 12 months, putatively via dysregulated apoptotic cell removal (Szondy *et al.*, 2003). Moreover, the lack of TG2 favours to the development of inflammatory stages, for example during liver injury (Nardacci *et al.*, 2003), and atherosclerosis (Van Herck *et al.*, 2010).

Phagocytosis deficiency caused by altered integrin signaling

Integrin signaling

Integrins are heterodimer receptors that contain non-covalently associated α and β subunits. There are approximately seventeen different α chains and eight different β chains, which pair together in specific patterns depending on the cell types in which they are expressed (Berton and Lowell 1999).

Integrins bind ECM components such as collagene, laminin, vitro-nectin, fibronectin and osteopontin or other membrane-bound receptors (Attur *et al.*, 2000). Most integrins initiate transmembrane signaling by activating focal adhesion kinase (FAK) and Src tyrosine kinases (SFKs) at adhesive sites (Galliher and Schiemann 2006).

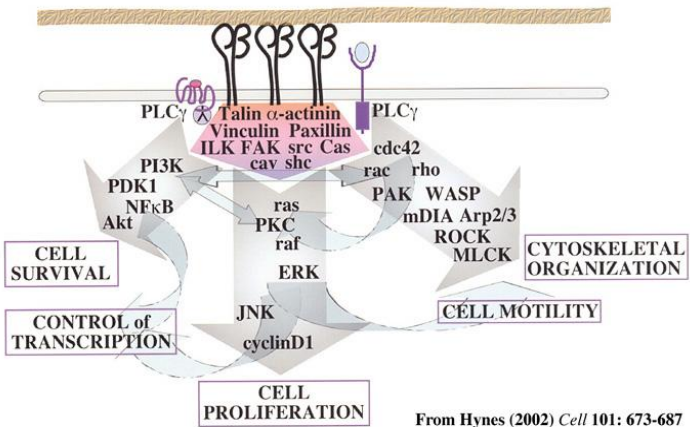


Figure 5. Integrin signaling *(Hynes, 2002)*

SFKs are constitutively associated with integrins, and their activation is a proximal and early consequence of integrin clustering (Arias-Salgado *et al.,* 2005). The presence of SFKs is obligatory for the majority of tyrosine phosphorylation events that follow integrin-mediated adhesion (Arias-Salgado *et al.,* 2005).

Upon integrin stimulation, FAK becomes activated and autophosphorylated at Tyr397 that confers the binding site for Src. This facilitates Src-mediated FAK phosphorylation on several tyrosine residues (Maa *et al.,* 2011) FAK and Src further recruit and activate various downstream effectors, such as PI3K/Akt, Ras/ERK, CrkII/Dock180/Rac (McLean *et al.*, 2005; Galliher and Schiemann 2006).

Interaction between TG2 and integrins

During monocyte differentiation into macrophages, high levels of integrin-bound surface TG2 is induced (Iismaa *et al.,* 2009). This correlates with macrophage phagocytotic capacity (Seiving *et al.,* 1991) as well as macrophage adhesion and migration (Akimov and Belkin 2001). During these processes, TG2 interacts with integrins of the β_1 and β_3 subfamilies (Akimov *et al.,* 2000, Akimov and Belkin 2001). These interactions induce integrin clustering and modify integrin signaling (Tóth *et al.,* 2009b). Moreover, integrin/TG2 complexes are detected inside the cell during biosynthesis and accumulate as coreceptors on the cell surface (Akimov *et al.,* 2000, Akimov and Belkin 2001). TG2 contributes to the formation of phagocytic portals by binding to integrin β_3, a known phagocytic receptor, and its bridgin gmolecule, MFG-E8 (Tóth *et al.,* 2009b).

In the absence of TG2 the AC-induced integrin β_3 signaling leads to impaired RhoG and Rac1 activation, and in this way, the cytoskeletal rearrangement is failed. As a compensatory response, TG2 null macrophages elevate the expression levels of both integrin β_3 and RhoG, and the cell adhesion-induced integrin β_3 signaling is enhanced (Greenberg *et al.,* 1991, Tóth

et al., 2009a). This is in line with the defective apoptotic cell clearance of $TG2^{-/-}$ macrophages (Szondy *et al.,* 2003; Iismaa et al 2009).

The lack of TG2 leads to enhanced inflammatory responses

TG2 intercts with NF-κB-mediated inflammatory processes

Increased TG2 activity is a common feature of several inflammatory diseases. TG2 is involved in enhanced inflammation by participating in an inflammatory loop with NF-κB -the "master switch" for inflammation-, reducing free IκBα, leading to the translocation of free NF-κB into the nucleus (Lee *et al.,* 2004). Since the TG2 promoter contains a NF-κB binding motif (Mirza *et al.,* 1997), TG2 is induced by NF-κB, completing the cycle. In addition to NF-κB, several inflammatory cytokines including IFN-γ TGF-β are known to upregulate TG2 (Sane *et al.,* 2007). The upregulation of TG2 by TGF-β leads to another positive feedback loop. In addition, TG2 effect post-translational modifications of phospholipase A_2, an enzyme that releases arachadonic acid from cell membrane glycerophospholipids, leading to the synthesis of inflammatory eicosanoids (Sane *et al.,* 2007).

TG2 also contributes to neutrophil migration into areas of inflammation. $TG2^{-/-}$ mice have reduced superoxide anion generation, but increased neutrophil phagocytic activity compared to wild type mice (Balajthy *et al.,* 2006).In addition TG2 interacting with IκB/p50:p65 complex can cause permanent NF-κB activation (Mann *et al.,* 2006).

1.5. RECOGNITION OF PATHOGENES

Innate immunity is the first-line defense of multicellular organisms that operates to limit infection after exposure to microbes. Invertebrates and vertebrates share a common ancestry for this defense system, illustrated by the striking conservation of the intracellular signaling pathways that regulate the rapid transcriptional response to infection (Irving et al., 2001).

Recognition of pathogens implicates a wide variety of PRRs specific for PAMPs, including TLRs, lectins, scavenger receptors, and integrins (Zeisel *et al.,* 2005). Among them, TLRs are critical for the development of innate immune responses. In addition to TLRs, other PRRs can play similar roles to detect the danger signals in the cytoplasm. The NLR family is the largest identified family of intracellular PRRs, which include both NODs and NALPs (Zhang and Mosser, 2008).

1.5.1. Toll-like receptors (TLRs)

The search for a LPS receptor led to the discovery of Toll receptors. The gene encoding Toll was discovered in the 1980s as a Drosophila gene that affected dorsal/ventral patterning in the embryo (Monick and Hunninghake, 2002). The TLRs are type I integral membrane glycoproteins, and based on considerable homology in the cytoplasmic region, they are members of a larger superfamily that includes the IL-1 receptor (Akira and Takeda 2004). Homologues of Toll were identified through database searches, and so far, 13 members of the TLR family have been identified in mammals (Akira and Takeda 2004; *Tabl. 5*).

TLRs have also been reported to recognise endogenous ligands -such as hyaluronan oligosaccharides, fibronectin fragments, fibronectin fragments, heat shock proteins, antibody-DNA complexes and high mobility group box (HMGB)-1- are thought to be released during necrotic cell death induced by tissue damage, stress factors or infection, resulting in the release of cell components that initiate an inflammatory response (Drexler *et al.,* 2008).

NAME	LIGAND(S)	LIGAND ORIGIN	CELL TYPES
TLR1	triacyl lipopeptides		MoC/MPh, BC, a subset of DCs
TLR2	glycolipids, lipopeptides, lipoproteins, lipoteichoic acids	bacteria	MoC/MPh, myeloid DC, MaC
	HSP70	host cells	
	zymosan	fungi	
TLR3	dsRNA, polyI:C	viruses	DC, BC
TLR4	LPS	G- bacteria	MoC/MPh, myeloid DC, MaC, intestinal epithelium
	heat shock proteins	bacteria and host cells	
	fibrinogen, heparan sulphate, hyaluronic acid	host cells	
TLR5	flagellin	bacteria	MoC/MPh, a subset of DCs, intestinal epithelium
TLR6	diacyl lipopeptides	Mycoplasma	MoC/MPh, MaC, BC
TLR7	ssRNS		MoC/MPh, plasmocytoid DC, BC
TLR8	ssRNS		MoC/MPh, a subset of DCs, MaC
TLR9	unmethylated CpG oligodeoxynucleotide DNA	bacteria	MoC/MPh, BC, plasmacytoid DC
TLR10	unknown	unknown	MoC/MPh, BC
TLR11	profilin	Toxoplasma gondii	MoC/MPh, liver, kidney, urinary bladder, epithelium
TLR12	unknown	unknown	neurons[↓]
TLR13	unknown	virus	neurons, ependymal cells, astrocytes

Table 3. Summary of mammalian TLRs.

BC: B-cells; DC: dendritic cells; LPS: lipopolysaccharide; MaC: mast cell; MoC/MPh: monocyte/macrophage

(Nasu and Narahara 2010; Shi et al., 2011; Mishra et al., 2008, Pifer et al., 2011, Sallustro et al., 2002)

1.5.2. Toll-like receptor 4 (TLR4) and its signalling pathways

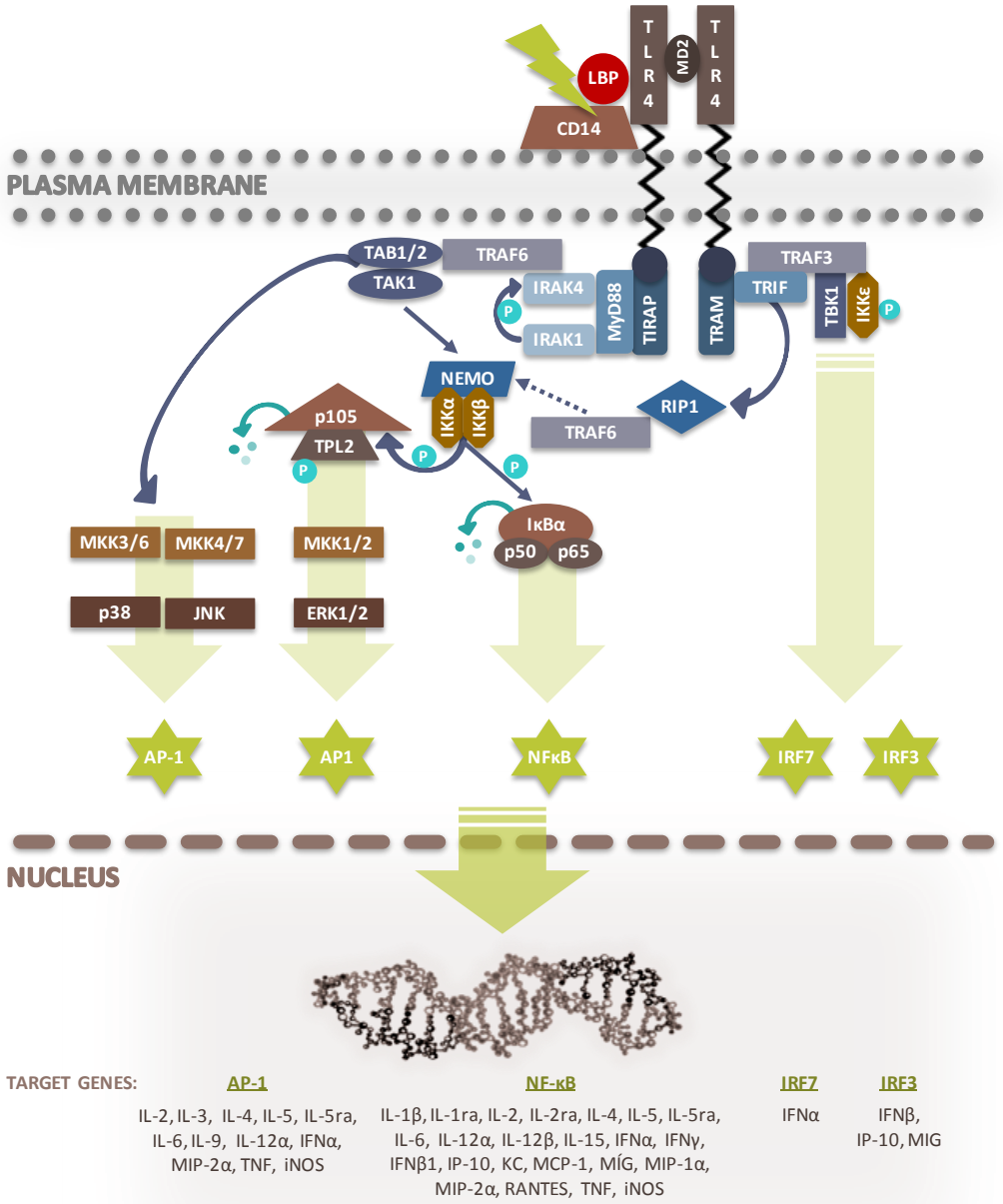
TLR4 (also designated as CD284) the signalling receptor for LPS, and is thus important in the activation of the innate immune system (Medzhitov *et al.*, 1997). Potent TLR4-dependent cell activation by LPS depends on sequential endotoxin—protein and protein—protein interactions with LBP, CD14, MD-2 and TLR4 (Gioannini *et al.*, 2005).

- The first host protein involved in the recognition of LPS is **LPS-binding protein** (LBP). LBP is an acute-phase protein, which circulates in the bloodstream where it recognizes and forms with lipid A moiety of LPS. The role of LBP appears to be that of aiding LPS to dock at the LPS receptor complex by initially binding LPS and then forming a ternary complex with CD14, thus enabling LPS to be transferred to TLR4 and MD-2 complex (Pålsson-McDermott and O'Neill 2004).
- **MD2** is a secreted glycoprotein that functions as an indispensable extracellular adaptor molecule for LPS-initiated signaling events by aiding in ligand recognition (Pålsson-McDermott and O'Neill 2004).
- **CD14** is a LPS receptor molecule, which acts as a co-receptor of TLR4. CD14 is found in two forms: a membrane-bound protein (mCD14) and as a soluble proteolytic fragment lacking the GPI anchor (sCD14) (Dauphinee and Karsan 2006).
- In addition to CD14, TLR4 and MD2, **other molecules** may be involved in CD14-independent signal initiation in macrophages, such as heat shock proteins, Hsp70 and Hsp90, CXCR4, and growth differentiation factor 5 (GDF5) (Triantafilou M and Triantafilou K 2002).

In addition to LPS, TLR4 also recognizes lipoteichoic acid, fibronectin, the fusion protein of respiratory syncytial virus (RSV) and taxol (Pålsson-McDermott and O'Neill 2004).

Canonical TLR4 signalling cascade

After ligand binding, TLRs dimerize and undergo the conformational change required for the downstream signalling (Akira and Takeda 2004). The homo- or heteromeric associations of cytoplasmic Toll/IL-1R (TIR) domain and TIR-containing adaptors (MyD88, TIRAP, TRIF, and TRAM) mediate canonical patway. MyD88 - an adaptor protein used by all TLRs except TLR3 - upon stimulation recruits IRAK to TLRs through interaction of the death domains of both molecules (Zeisel *et al.,* 2005). IRAK is activated by phosphorylation and then associates with TRAF6 (Takeda and Akira 2004). Upon TLR activation, TRAF6 is recruited to the receptor complex, and activated by IRAK-1 that binds to the TRAF domain of TRAF6. Then, the IRAK-1/TRAF6 complex dissociates from the receptor and associates with TAK1, TAB1 and TAB2, at the membrane portion (Qian *et al.,* 2001). IRAK-1 stays in the membrane and is degraded, whereas the complex of TRAF6, TAK1, TAB1, and TAB2 moves into the cyto plasm, where it forms a large complex with other proteins, such as the E2 ligases Ubc13 and Uev1A (Deng *et al.,* 2000). This complex induces the activation of TAK1. Activated TAK1 phosphorylates the IKK complex, which consists IKKα, IKKβ, and NEMO (also known as inhibitor IKK-γ) (Takeda and Akira 2004). The IκBα protein inactivates the NF-κB transcription factor by masking the nuclear localization signals of NF-κB proteins and keeping them sequestered in an inactive state in the cytoplasm (Mercurio *et al.,* 1997, Rothwarf et al 1998). IKK specifically phosphorylates the inhibitory IκBα protein (Karin 1999). This phosphorylation results in the dissociation of IκBα fromNF-κB and thereby activates NF-κB (Régnier *et al.,* 1997, Jacobs and Harrison 1998). Moreover IKK can phosphorylate NF-κB1 p105, triggering its degradation. This liberates TPL-2 kinase (MAPKKK8, MAP3K8) from p105 inhibition, facilitating phosphorylation of MEK, which then phosphorylates and activates JNK and MAPK pathway (Beinke *et al.,* 2003). Other molecules, such as TOLLIP (O'Neill 2000), the pellinos (Sun *et al.,* 2008), PI3K, Akt (Fang 2011, Kacimi *et al.,* 2011), ECSIT (West *et al.,* 2011) p38, CREB (Park *et al.,* 2005) and the SRC-family tyrosine kinases also also may be involved in the canonical TLR4 patway.



AP-1	activator protein 1
Erk	extracellular regulated MAP kinase
IκB	NF-κB inhibitor α
IKKs	IκB kinases
IRAKs	IL-1R-associated kinases
IRFs	interferon regulatory factors
JNK	c-Jun N-terminal kinase
LBP	lipopolysaccharide-binding protein
MAPK	mitogen-activated protein kinase
MD2	myeloid differentiation factor 2
MKKs	MAPK kinases (also known as MAP2K)
MyD88	myeloid differentiation primary-response protein 88
NEMO	NF-κB essential modulator
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
p105	NF-κB1 subunit p105
p38	p38 MAPK
RIP1	receptor-interacting protein 1
TABs	TAK1-binding proteins
TAK1	transforming growth factor-κ-activated kinase
TBK	TANK-binding kinase
TIRAP	TIR-domain-containing adaptor protein *(also known as MAL)*
TLR4	Toll-like receptor 4
TPL2	tumour progression locus 2
TRAFs	tumour-necrosis factor-receptor-associated factors
TRAM	TRIF-related adaptor *molecule (also known as TICAM2)*
TRIF	TIR-domain-containing adaptor protein inducing IFNβ *(also known as TICAM1)*

Figure 6. The TLR4 signaling pathway

(see references in the text)

MyD88-independent TLR4 pathway

Closer study of MyD88-deficient cells has revealed the existence of MyD88-dependent and -independent pathways, both of which mediate signalling in response to LPS (Akira and Takeda 2004). MyD88-independent pathways, involving TRIF are used by TLR4 and TLR3, together with TRAM in the case of TLR4 (Zeisel *et al.,* 2005). The recruitment of these adaptor molecules to the receptor complex results in binding of TRAF6 and RIP1 to TRIF, leading to the late-phase activation of NF-κB, which contributes to the induction of IFN-β (Dauphinee and Karsan 2006). In a parallel pathway, with the help of TRAF3, TANK, TBK1 and IKKε TLR4 interacts with TRIF and mediate activation of IRF3 (Dauphinee and Karsan 2006) and IRF7, which leading to the induction of IFNβ and IFN-α1 respectlively (Fitzgerald *et al.,* 2003). A number of genes known to be IFN-inducible genes were identified, such as glucocorticoid-attentuated response gene 16 (GARG16), immunoresponsive gene 1 (IRG1) and the gene encoding CXCL10 (also called as IP10). As expected, genes encoding inflammatory cytokines, such as TNF, IL-6 and IL-1β, were not expressed (Akira and Takeda 2004).

Feedback regulation

So far, IKKα, IκBδ, IRAK-M, the deubiquitinating enzyme Cezanne, SOCS1 and SOCS3, tripartite-motif-containing 30-α (TRIM30-α), cylindromatosis (CYLD), phosphoinositide-dependent kinase 1 (PDK-1), NFκB–inducing kinase (NIK) and the PI3K signaling cascade have been shown to participate in the LPS-induced IL-6, TNF-α and IL-12 production in an autoregulatory feedback loop (Chaurasia *et al.,* 2010; Shih *et al.,* 2009; Enesa *et al.,* 2008; Razani *et al.,* 2010). Another negative feedback loop operating at the transcriptional level involves ATF3, a member of the CREB/ATF family of transcription factors (Chawla, 2010), and miRNAs (e.g. miR-155, miR-21, miR-146a/b etc.) (Imaizumi *et al.,* 2010; Marquez *et al.,* 2010; Bhaumik *et al.,* 2009). The release of soluble anti-inflammatory mediators, such as TGF-β, IL-10 and PGE2 also contributes to control of inflammatory responses (Imai *et al.,* 1999; Berlato *et al.,* 2002; Vassiliou *et al.,* 2003).

1.5.3. The influence of integrins on TLR4-mediated inflammatory response

Although originally identified as adhesion molecules, integrins are now known to mediate a wide variety of signaling functions, and consequently, integrins influence many biologic systems. They are involved in hematopoiesis, hemostasis, immune regulation, and the inflamma-

tory response (Yonekawa *et al.,* 2005). The importance of integrin-dependent processes in inflammatory and immune responses of leukocytes is illustrated by several *in vitro* and *in vivo* studies on tissue leukocytes:

- In mice, blockade of α_4 on intrapulmonary leukocytes decreased allergen-induced lung inflammation (Henderson *et al.,* 1997).
- Blockade of $\alpha_1\beta_1$significantly ameliorates inflammatory responses in animal models of arthritis, colitis, influenza infection, and asthma (Yonekawa *et al.,* 2005).
- ITG $\alpha_v\beta_3$ ligation results in sustained increases of the transcription factor NF-κB DNA-binding activity, which leads to raise in TNF-α, IL-1β, IL-6 and IL-8, but to reduction in IL-10 mRNA level (Antonov *et al.,* 2011).
- Synthetic peptides containing RGDS (Arg-Gly-Asp-Ser) sequence for ITG α_v inhibit inflammatory responses to intratracheal LPS treatment and the integrin signaled MAPK pathway during the development of acute lung injury (Moon *et al.,* 2009).

These observations demonstrate that, in addition to the TLR receptor complex, optimal LPS signaling requires complementary signals from integrin receptors (Monick *et al.,* 2002).

Src tyrosine kinases (SFKs)

The members of nonreceptor tyrosine kinase family (SFKs: Src, Fyn, Yes, Fgr, Hck, Lck, Lyn, Blk, and Yrk), are not only important transducers of integrin signaling, but also participate in cytokine signaling and inflammatory responses (Lee *et al.,* 2007).

The ligation of TLR4 activates SFKs, which phosphorylating tyrosines in different signalling proteins (Gong *et al.,* 2008) leads to the induction pro-inflammatory cytokine genes via NFκB- or AP-1-mediated pathways (Smolinska *et al.,* 2008). In macrophages, Src phosphorylates and activates IKKβ, which leads to the degradation of IκBα, a negative regulator of NF-κB activation (Baldwin 1996).

2. AIMS OF THE STUDY

We have previously reported that $TG2^{-/-}$ mice develop an age-dependent autoimmunity due to defective *in vivo* clearance of apoptotic cells (Fig.7) (Szondy *et al.*, 2003). It is very likely that altered pro-inflammatory cytokine production by macrophages engulfing ACs contributes to the phenomenon, since $CD14^{-/-}$ mice, which show clear defect in the *in vivo* clearance of ACs, but no alteration in pro-inflammatory cytokine production during engulfment of ACs, do not develop autoimmunity (Devitt *et al.*, 2004). In contrast, macrophages from TG2, Mer tyrosine kinase or C1q null mice, which show also a defect in the *in vivo* clearance of ACs, but develop autoimmunity, produce elevated levels of pro-inflammatory cytokines, when engulf ACs (Falasca *et al.*, 2005; Tibrewal *et al.*, 2008; Fraser *et al.*, 2006).

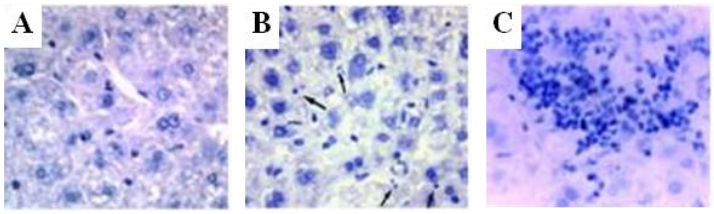


Figure 7. Morphological changes occurring in liver of WT and TG2 null mice after $PbNO_3$ treatment *(Szondy et al., 2003)*

Light microscopy of treated livers after 5 days. H&E-stained sections (×63) demonstrate the presence of numerous apoptotic hepatocytes (arrows) and massive inflammatory infiltrate in $TG2^{-/-}$ (B and C), but not in WT liver (A).

Since the activation of TGF-β − which not only mediates the anti-inflammatory effects of ACs but also takes a part in the resolution of LPS-induced inflammatory responses − is defective in the absence of TG2, it is very likely that the lack of TGF-β is responsible for the enhanced inflammatory responses of TG2 null macrophages. With our present study we wanted to test this hypothesis. Our questions were:

1. Is the lack of active TGF-β responsible for the enhanced pro-inflammatory cytokine production of TG2 null macrophages?

2. Are there any other TGF-β-independent mechanism involved in the increased LPS-sensitivity of macrophages lacking TG2?

Adenosine has been known for a long time to mediate anti-inflammatory effects on macrophages via its A_{2A} receptor subtype. Since our previous results have challenged the central and exclusive role of TGF-β in the immunosuppressive effect of AC uptake, we concentrated to the following issues:

1. Is it possible that adenosine is one of the soluble anti-inflammatory mediators released by macrophages in response to apoptotic cell exposure?
2. If so, does $A_{2A}R$ mediate the anti-inflammatory signal of adenosine during apoptotic cell clearance in macrophages?

3. MATERIALS AND METHODS

3.1. Reagents

All reagents were obtained from (Sigma-Aldrich, Budapest) except indicated otherwise.

NAME	ACTION		NAME	ACTION
CGS21680	selective $A_{2A}R$ agonist		**SCH442416**	selective $A_{2A}R$ antagonist
22(R)-OHCh	LXR agonist			
GW501516	PPARδ agonist			
Actinomycin D	Transcription inhibitor			
Cycloheximide	Protein synthesis inhibitor			
Cytochalasin D	Actin polimerization inhibitor			
Db-cAMP	PKA activator		**Rp-cAMP**	PKA inhibitor
CTX	$[cAMP]_{IC}$ ↑			
Forskolin	adenylyl cyclase activator			
L-NAME	non-selective NOS inhibitor		**SNP**	NO donor
PP2	Src family kinase inhibitor			
R294	TG2 inhibitor			

Table 4. Summary of applied drugs.

3.2. Animals

Most of the experiments were done using 3 months old male wild type (C57B/6 and FVB), $A_{2A}R$ deficient mice (Ledent *et al.*, 1997) generated on an FVB background; and TG2 deficient mice (Boisvert *et al.*, 2006) generated on C57B/6 background. Some of the experiments were also carried out on PPARδ (Mukundan *et al.*, 2009) or LXR (A-Gonzalez *et al.*, 2009) deficient mice generated on 129 SvJ, or on a mixed background of C57Bl/6 and 129Sv, respectively. These studies have been reviewed and approved by the review committee of the University of Debrecen (DEMÁB).

3.3. Macrophage isolation and culturing

Macrophages were obtained by peritoneal lavage with sterile physiological saline. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 for 2 days before use. After 3-4 hours of incubation, the non-adherent cells were

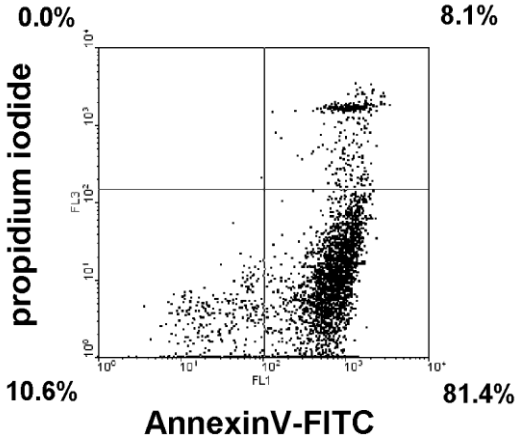
washed away. Before the experiments, the cells were cultured for 2 days replacing media daily. For bone marrow-derived macrophages, wild type and PPARδ null bone marrow was isolated from femurs, and cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin and 10% L929 conditioned media for 10 days. The non-adherent cells were washed away from the 3rd day daily.

3.4. Adenoviral gene delivery system

Recombinant, replication-deficient adenoviral vectors encoding either LacZ and the murine TG2 gene or the secretion deficient (TG2-S), guanine nucleotide binding deficient (TG2-G1 and TG2-G2) or crosslinking function deficient (TG2-X) TG2 mutants were produced using the AdEasy XL system (Stratagene) according to the manufacturer's instruction. Virus titers were determined by plaque assay in 293 cells after exposing them to virus for 48 hours in DMEM medium supplemented with 2% serum and antibiotics. For gene delivery, $2x10^6$ macrophages were exposed to $2x10^9$ PFU/ml virus particles for 48 hrs in the same medium. LacZ expression was determined with X-gal staining, while TG2 expressions by Western blot analysis using anti-TG2 specific antibodies.

3.5. Thymocyte apoptosis induction *in vitro*

ACs were prepared from wild-type mice in all cases. Thymocytes isolated from 4 weeks old mice were cultured for 24 hrs. (10^7 cells/ml) in the absence of serum (RPMI 1640 medium supplemented with penicillin/streptomycin).



This method typically resulted in >80% ACs (as assessed by propidium iodide/AnnexinV-FITC staining, Fig. 8).

Figure 8. FACS analysis of serum-starved thymocytes.
(AnnexinV-FITC and propidium iodide staining)

In case of NB4 cells, the apoptosis was induced by 10 μM As_2O_3 –treatment for 12hrs (Chen *et al.*, 1997). ACs were used at a 10:1 (AC: macrophage) ratio.

3.6. Preparation of the cell culture medium for adenosine measurement

The experiments were performed by co-incubating $A_{2A}R^{+/+}$ macrophages (1 x 10^6 cells/sample) with ACs in 1:10 ratio. For the respective experiments, macrophages were pre-treated with 50 mM cytochalasin D for 1 hr. to block the phagocytic activity of macrophages. After 2 hours of phagocytosis, the supernatants were replaced with fresh culture media. After 5 hours of incubation (at 37°C) the supernatants were collected, deproteinized with 5 ml ice cold 0.6 N $HClO_4$ and stored on -80°C. The determination of ADO concentration was carried out with a reverse phase HPLC method as described (Delbar *et al.*, 1999).

3.7. Flow cytometry

Wild type and $A_{2A}R$ null peritoneal macrophages were coincubated with apoptotic thymocytes for one hr. in 1:10 ratio. After replacing media and washing away the ACs, macrophages were incubated for additional one, three or five hrs. For some experiments, all these treatments were carried out in the presence of 5 μg / ml actinomycin D, or 0.1 μg / ml cycloheximide. For characterizing the regulation of the expression of the receptor macrophages were treated with various concentrations of 22-R(OH)-cholesterol, an LXR agonist, or GW501516, a PPARδ agonist (Glaxo Smith Kline), for 3 hrs. After the treatments macrophages were washed (1xPBS), collected, blocked with 50% FBS for 30 min. and labeled with anti-mouse $A_{2A}R$ antibody (BD Pharmingen) or goat IgG isotype control. For the detection, cells were stained with FITC-conjugated anti-goat IgG.

In case of TLR4 and CD14 determination, $5x10^5$ peritoneal macrophages were labeled in 50 μl PBS with FITC conjugated anti-CD14 antibody (Pharmingen) or rabbit-anti mouse TLR4 antibody (Santa Cruz Biotechnology) washed with PBS and incubated further with FITC-anti-rabbit antibody. Stained cells were analyzed on a FACSCalibur (BD Biosciences). The results were analyzed by WinMDI 2.9 software.

3.8. Determination of ADORA2, IL-6 and TNF-α mRNA expression

Wild type, $A_{2A}R$ null and LXR null peritoneal, or PPARδ wild type and knock out bone marrow derived macrophages were coincubated with LPS (various target cell types: apoptotic, living, heat killed (45 min, 55°C) or anti-CD3-pretreated (10 μg/ml, 20 min; R&D Systems) thymocytes for 1 hr in 1:10 ratio. After washing away the ACs and replacing media, mRNA was collected 2 hours later.

3.9. Determination of cytokine production

Wild type and $A_{2A}R$ null peritoneal macrophages were plated onto 24-well plates at a density of $5x10^5$ cells/well. To determine cytokine production by macrophages exposed to ACs, macrophages were exposed to ACs for one hr in the presence or absence of an $A_{2A}R$-selective antagonist SCH442416 (10 nM, Tocris) or $A_{2A}R$-selective agonist CGS21680 (10 nM). ACs than were washed away, SCH442416 or CHS21680 were re-added and the macrophages were cultured for an additional five hours. At the end of culture cell culture media were analyzed by Mouse Cytokine Array (Proteome Profile Array from R&D Systems). The pixel density in each spot of the array was determined by Image J software.

	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24
A	+ control											+ control
B	BLC	C5a	G-CSF	GM-CSF	I-309	Eotaxin	sICAM-1	IFN-γ	IL-1α	IL-1β	IL-1ra	IL-2
C	IL-3	IL-4	IL-5	IL-6	IL-7	IL-10	IL-13	IL-12 p70	IL-16	IL-17	IL-23	IL-27
D	IP-10	I-TAC	KC	M-CSF	JE	MCP-5	MIG	MIP-1α	MIP-1β	MIP-2	RANTES	SDF-1
E	TARC	TIMP-1	TNF-α	TREM-1								
F	+ control											- control

Table 5. The map of 40 cytokines detected on cytokine array membranes.

Alternatively, cytokine-induced neutrophil-attracting chemokine (KC), TGF-β,. macrophage inflammatory protein-2 (MIP-2) and IL-10 cytokine levels were measured with R&D Systems ELISA kits.

To determine the effective amount of CGS21680 and SCH442416 for the Cytokine Array wild-type peritoneal macrophages ($5x10^5$ cells/sample) were treated with increasing amount of agonist, or with increasing amount of antagonist in combination with 10 nM agonist. After 30 min, pretreatment MIP-2 production was triggered by the addition of 200 ng/ml bacterial lipopolisaccharide (LPS). After 1 hr incubation LPS was washed away, the CGS21680 and

SCH442416 were re-added and the macrophages were cultured for an additional five hrs. The MIP-2 levels were determined by ELISA from cell culture media (Fig.9).

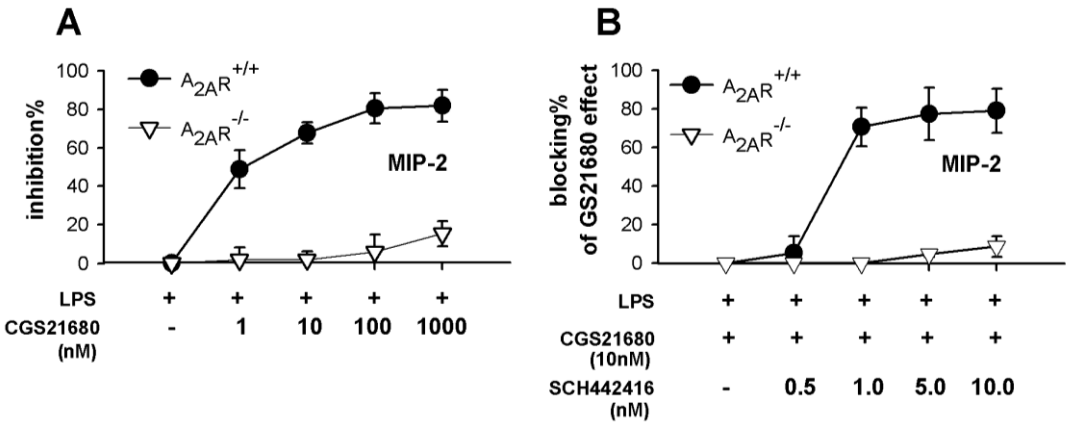


Figure 9. Titration of CGS21680 and SCH442416.

In case of CGS21680 treatment the percentages of inhibition were calculated by comparing the MIP-2 levels in the agonist treated samples to the LPS control (LPS control= 0% inhibition). In the SCH442416 titration we blocked the inhibitory effect of agonist CGS21680 (10 nM) with increasing amount of antagonist. The efficiency represented by blocking percentages, where the MIP-2 amounts in the antagonist treated samples were compared to the MIP-2 levels in the agonist control (LPS+CGS21680= 0% inhibition). Results are expressed as mean ± S.D. of three independent experiments.

In case of TG2 experiments wild-type and TG2 null peritoneal macrophages were seeded onto 24-well plates at a density of $5x10^5$ cells/well in 500 µl medium. Cell were treated with 100 ng/ml LPS (Sigma) for one hour in the presence or absence of increasing concentrations of recombinant TGF-β (Serotec) as indicated in the results section. After one hour LPS was removed and fresh medium was added to the cells containing either recombinant TGF-β, 4 ng/ml neutralizing anti-pan TGF-β antibody (R&D Systems), isotype control antibody or vehicle. Supernatants were collected and frozen at the indicated time points. IL-6, TNF-α, and active TGF-β cytokine levels were measured with R&D Systems ELISA kits.

3.10. Quantitative PCR and quantitative RT-PCR

After various treatments, 3 x 10^6 peritoneal macrophages were washed with ice-cold PBS. RNA was extracted with Tri-reagent. cDNA was synthesized with High-Capacity cDNA Archive Kit (Applied Biosystems) on ABI 2720 Thermal Cycler (Applied Biosystem).

Cyclophilin, MIP-2, ADORA2, iNOS, eNOS, IL-6, TNF-α, ArgI and ArgII levels were determined with Taq-Man PCR using FAM-GMB-labelled primers (Applied Biosystems). Samples were run in triplicates on ABI Prism 7900 using ABM Prism SDS2.1 software for evaluation (Applied Biosystems). Gene expression was normalized to cyclophilin expression.

REVERSE TRANSCRIPTION		RT-qPCR	
COMPONENT	Volume/sample	COMPONENT	Volume/sample
Nuclease free water	4.2 µl	Nuclease free water	2.115 µl
RT buffer (10x)	2.0 µl	Buffer (10x)	1.0 µl
		MgCl2 (25 mM)	1.2 µl
dNTP mix (100 mM)	0.8 µl	dNTP mix (2.5 mM)	0.5 µl
Random primers (10x)	2.0 µl	20x oligo mix (ABI)	0.25 µl
MultiScribe RT	1.0 µl	Taq polimerase (5 U/µl)	0.063 µl
RNA	10.0 µl	cDNA	5.0 µl
TOTAL	20 µl	TOTAL	10.003 µl
THERMAL PROFILE			
Step 1	25°C/10 min	Denaturation	94°C/1 min
Step 2	37°C/120 min	Hybridization	94°C/12 sec
Step 3	85°C/5 min	Elongation	60°C/30 sec
Step 4	4°C/∞	Repeat	40 cycles

Table 6. Details of reverse transcription and RT-qPCR measurement.

Calculation:

$$\textbf{relative gene expression: } \frac{2^{(Ct_{max} - Ct_{gene\ of\ interest})}}{2^{(Ct_{max} - Ct_{cyclophilin})}}$$

$Ct_{max}=40$

Ct: threshold cycle

3.11. mRNA stability determination

Wild-type and TG2 null peritoneal macrophages were pre-treated with 100 ng/ml LPS for 1 h followed by addition of 1 µg/ml Actinomycin D (Sigma). Total RNA was isolated at the indicated time points and TNF-α mRNA was measured by quantitative RT-PCR.

3.12. Phagocytosis assay

For phagocytosis assays, macrophages were stained overnight with10 µM 5-(and 6-)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMTMR) (Invitrogen), while thymocytes were labeled overnight with 6 µM 6-carboxy-3 ,6 -diacetylfluorescein (CFDA) (Invitrogen). Macrophages were incubated with apoptotic thymocytes in 10:1 target/macrophage ratio for one hr. Cells incubated with apoptotic thymocytes incubated at 4°C were used as controls. After washing, the cells on the plate were trypsinized, resuspended in cold medium with 0.5%

sodium azide, and 10,000–20,000 cells were analyzed for each point by two-color flow cytometry (FACSCalibur, BD Biosciences).

For visualizing ACs in macrophages, macrophages were plated in 2-well chamber slides in a concentration of $5x10^5$/well and cultured for 48 h before CMTMR staining. After coculturing macrophages with CFDA labeled ACs for 30 min, cells were washed and fixed in ethanol/acetone (1:1) for 10 min at -20°C. Images were taken with a Olympus FV1000 confocal laser scanning microscope. 500 cells were counted for AC uptake in each individual experiment.

3.13. Determination of NO production of macrophages engulfing apoptotic cells

$A_{2A}R^{+/+}$ or $A_{2A}R^{-/-}$ macrophages were exposed to ACs for 1 hr. Media were replaced and macrophages were incubated for an additional 1 hr. Cell culture supernatants were analyzed for NO. Nitrite concentration in the samples was then measured by the Griess reaction (Kleinbongard *et al.,* 2002) by adding 100 µl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 µl samples of medium. The optical density at 550 nm (OD550) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, Calif.). Nitrate concentrations were calculated by comparison with OD550 of standard solutions of sodium nitrate prepared in culture medium.

3.14. *In vivo* neutrophil migration assay

$A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice were injected with 2 ml of 4% thioglycollate intraperitoneally. 4 days later they were injected intraperitoneally with 2×10^6 of ACs suspended in 2 ml of physiological saline or 2 ml physiological saline. After 3 hours the peritoneal cells were collected, washed, blocked with 50% FBS and stained with FITC-conjugated rat anti-mouse Gr-1 (RB6-8C5) (Pharmingen), or V450 rat IG2bκ isotype control for 30 min. The detection was carried out using FITC conjugated anti-rat IgG (Pharmingen). Cells were then washed, fixed and analyzed by flow cytometry (FACSCalibur, BD Biosciences) to determine the percentages of neutrophils in the total cell population. In some experiments rat anti–mouse KC (clone 48415.111; IgG2a), rat anti–mouse MIP-2 (clone 40605; IgG2b) or their isotype controls IgG2a, (clone 5444.11), and IgG2b (clone 141945) obtained from R&D Systems were injected together with the ACs into mice.

3.15. Western blot

Wild type and TG2 null peritoneal macrophages were seeded onto 6-well plates at a density of $2x10^6$ cells/well. Cells were treated with 100 ng/ml LPS for the indicated time periods, pre-treated with 1μg/ml soluble vitronectin (Sigma) for 30 minutes or pretreated with 2 μM PP2 (Calbiochem) for 24 hours. Cells were harvested at the indicated time points and boiled 2x sample buffer and loaded onto SDS PAGE gels. PVDF membranes were probed with anti-IκKβα (Santa Cruz Biotechnology), anti-cSrc (Santa Cruz Biotechnology), anti-phospho(Tyr416)-Src (Cells Signaling Technology), anti-integrin β_3 (MBL International), anti-phospho (Tyr474) integrin β_3 (Santa Cruz Biotechnology) anti-TG2 (Santa Cruz Biotechnology) and β-actin antibodies (Sigma).

3.16. Determination of NK-κB p65 nuclear translocation

10^7 wild type and TG2 null peritoneal macrophages were treated with 100 ng/ml LPS for 30, 60 and 120 minutes. Cell were rinsed with ice cold PBS and nuclei were isolated with Nuclei EZ kit (Sigma) according to manufacturer's instruction. Nuclear p65 subunit was detected with TransAM p65 kit (ActiveMotif) according to manufacturer's instruction.

3.17. Statistical analyses

All the data are representative of at least three independent experiments carried out on three different days. Values are expressed as mean ± S.D. P values were calculated by using two-tailed Student's t-test for two samples of unequal variance. The analysis of cytokine array experiments was carried out by ANOVA test. A single asterisk ($P < 0.05$) indicates statistical significance.

4. RESULTS

4.1. TG2 INFLUENCING $\alpha_v\beta_3$ INTEGRIN-MEDIATED SRC SIGNALING HAS AN EFFECT ON THE INFLAMMATORY RESPONSE OF MACROPHAGES

4.1.1. TG2 null macrophages respond by elevated pro-inflammatory cytokine production to LPS treatment

To determine the time course of LPS-stimulated pro-inflammatory cytokine production of wild type resident peritoneal macrophages, macrophages were exposed to 100 ng/ml LPS, and the LPS-induced IL-6 and TNF-α production was determined by ELISA at various time points (Fig.10).

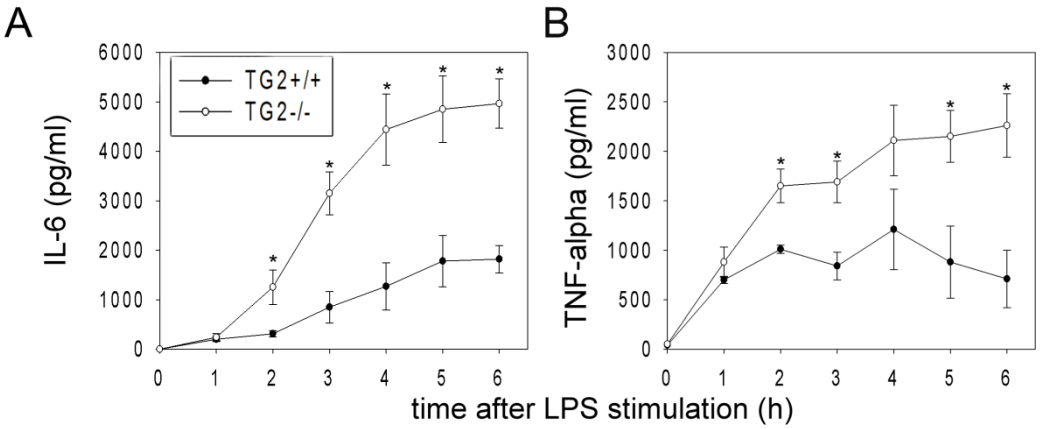


Figure 10. LPS exposed TG2 null macrophages produce higher amounts of TNF-α and IL-6 than wild-type cells.

Wild type (filled circles) and TG2 null (open circles) peritoneal macrophages were treated for 1h with 100 ng/ml LPS. After incubation LPS was washed away and fresh medium was added to the cells. Supernatants were collected at the indicated time points and kept at -20^oC until analysis. IL-6 and TNF-α cytokine levels were determined by ELISA technique. The results are representative of four independent experiments and are shown as mean ± SD. ($p<0.05$).*

To avoid detection of *in vivo* induced pro-inflammatory cytokine production, macrophages were plated for 2 days, by a time when no more endogenous cytokine production was found, before addition of the LPS. As shown in Fig.10, the IL-6 and TNF-α production of wild-type peritoneal macrophages reached their maximum at 6h following LPS stimulation, and these levels remained with no significant alterations during the following 20 hours (data not shown).

While IL-6 production started to raise only after one hour of LPS stimulation (Fig.10A), a significant amount of TNF-α was detected already at one hour of LPS stimulation (Fig.10B) indicating that some TNF-α is stored within the macrophages, which is released upon LPS-stimulation. Peritoneal macrophages responded with more IL-6 than TNF-α production to LPS stimulation. Loss of TG2 did not affect the kinetics of IL-6 or TNF-α production , but the levels of pro-inflammatory cytokines produced by TG2 null macrophages were higher at each time points as compared to that produced by the wild-type ones. These data indicate that in the absence of TG2 macrophages are more sensitive to LPS stimulation than their wild type counterparts.

4.1.2. Elevated pro-inflammatory cytokine production by TG2 null macrophages is not related to the lack of TGF-β activation

Since TG2 is required for the activation of latent TGF-β produced by macrophages (Nunes *et al.*, 1995), and active TGF-β has been proposed to act as an autocrine feed back regulator of pro-inflammatory cytokine production of LPS-stimulated macrophages (Letterio and Roberts,

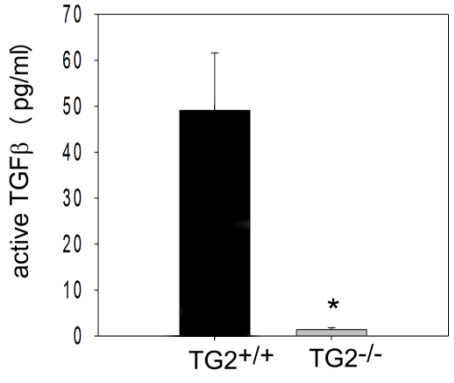


Figure 11. The enhanced pro-inflammatory cytokine production of LPS-stimulated TG2 null macrophages is not related to the lack of TGF-β activetion.

*Wild-type (black bars) and TG2 null (grey bars) peritoneal macrophages were treated for 1h with 100 ng/ml LPS After 1h incubation LPS was washed away, but the indicated compounds were re-added in the fresh medium. Supernatants were collected and frozen after 5h. Active TGF-β levels were determined by ELISA technique. The results are representative of three independent experiments are shown as mean ± SD. (*p<0.05).*

1998), we decided to test whether the enhanced pro-inflammatory cytokine production is related to the lack of TGF-β activation by TG2 null macrophages. As shown in Fig.11 in line with the previously reported data about the requirement of TG2 for the activation of latent TGF-β (Nunes *et al.,* 1995), TG2 null macrophages indeed were unable to produce detectable amounts of active TGF-β.

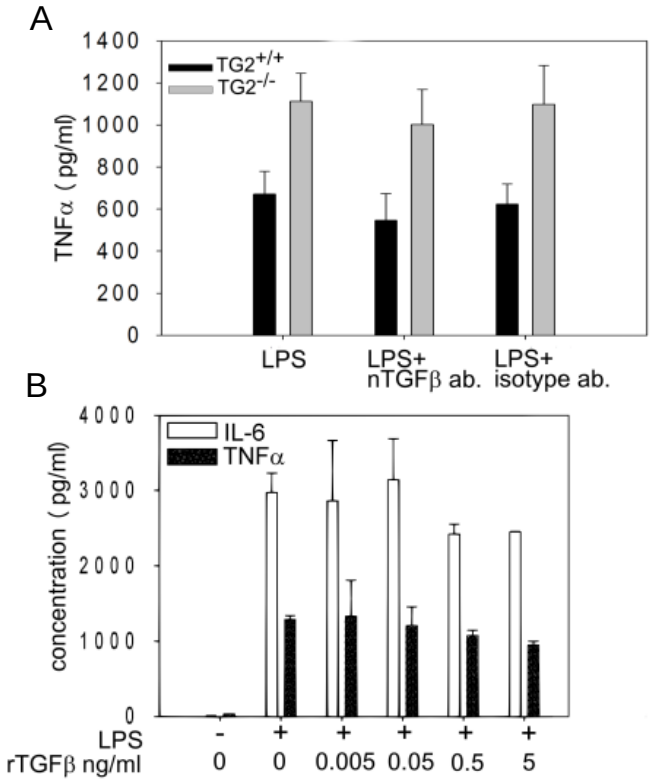


Figure 12. The enhanced pro-inflammatory cytokine production of LPS-stimulated TG2 null macrophages is not related to the lack of TGF-β activation.

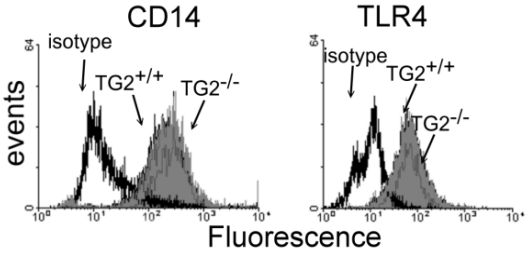
*Wild-type (black bars) and TG2 null (grey bars) peritoneal macrophages were treated for 1h with 100 ng/ml LPS in the presence of 4 ng/ml neutralizing anti-TGF-β (A) or its isotype control antibody. (B) In addition, TG2 null macrophages were also treated by LPS in the presence of increasing amounts of recombinant TGF-β. After 1h incubation LPS was washed away, but the indicated compounds were re-added in the fresh medium. Supernatants were collected and frozen after 5h. Active TGF-β, TNF-α, and IL-6 cytokine levels were determined by ELISA technique. The results are representative of three independent experiments are shown as mean ± SD. (*p<0.05).*

However, a pan TGF-β neutralizing antibody (Denning *et al.,* 2007) failed to enhance the LPS-induced early pro-inflammatory cytokine production (Fig.12A).The neutraliZing antibody concentration used was sufficient to block all the active TGF-β, because after addition of the neutralising antibody no active TGF-β was detectable in the supernatants by ELISA (data not shown). In addition, recombinant TGF-β, in the concentrations we detected active TGF-β in the cell culture medium, could not significantly affect LPS-induced pro-inflammatory cytokine production of TG2 null macrophages (Fig.12B), indicating that TGF-β

has no effect on the short-term LPS-induced cytokine formation of macrophages, and not the lack TGF-β production is responsible for the enhanced pro-inflammatory cytokine formation by TG2 null macrophages. So we decided to test further the characteristics of LPS signaling in TG2 null peritoneal macrophages.

4.1.3. Basal levels of IκBα are decreased in TG2 null macrophages

LPS is recognized by macrophages via TLR4 together with the accessory molecule CD14 (Kawai and Akira, 2006). To test, whether the expression of the LPS sensing receptors have changed in TG2 null macrophages, the cell surface expression levels of CD14 and TLR4 were detected by flow cytometry. However, as shown in Fig.13, no change in the cell surface expression of these receptors was found in TG2 null macrophages, indicating that not an altered expression of these receptors is responsible for the enhanced LPS sensitivity.



Flow cytometric analysis of cell surface CD14 and TLR4 expression of wild type and TG2 null peritoneal macrophages. Open histograms on the left indicate isotype controls.

Figure 13. The lack of TG2 does not affect the cell surface level of LPS receptor.

There are several levels, at which LPS-induced production of TNF-α and IL-6 can be controlled. First, we decided to test whether their transcription is altered. As shown in Fig.14A, using Q-PCR technique we could not detect basal levels of mRNA for the two pro-inflammatory cytokines. Exposure to LPS enhanced the expression of TNF-α and IL6 in both types of macrophages, but the TG2 null macrophages showed about two and four fold higher mRNA productions, respectively. To differentiate whether the transcription or the stability of the mRNA was altered in the absence of TG2, LPS-stimulated macrophages were exposed to actinomycin D, a transcription inhibitor and the time dependent decrease in the mRNA expression of TNF-α was followed in both wild-type and TG2 null macrophages (Fig.14B). Since no change in the kinetics of the mRNA degradation was found, it is likely that the loss of TG2 alters the pro-inflammatory cytokine production at transcriptional level.

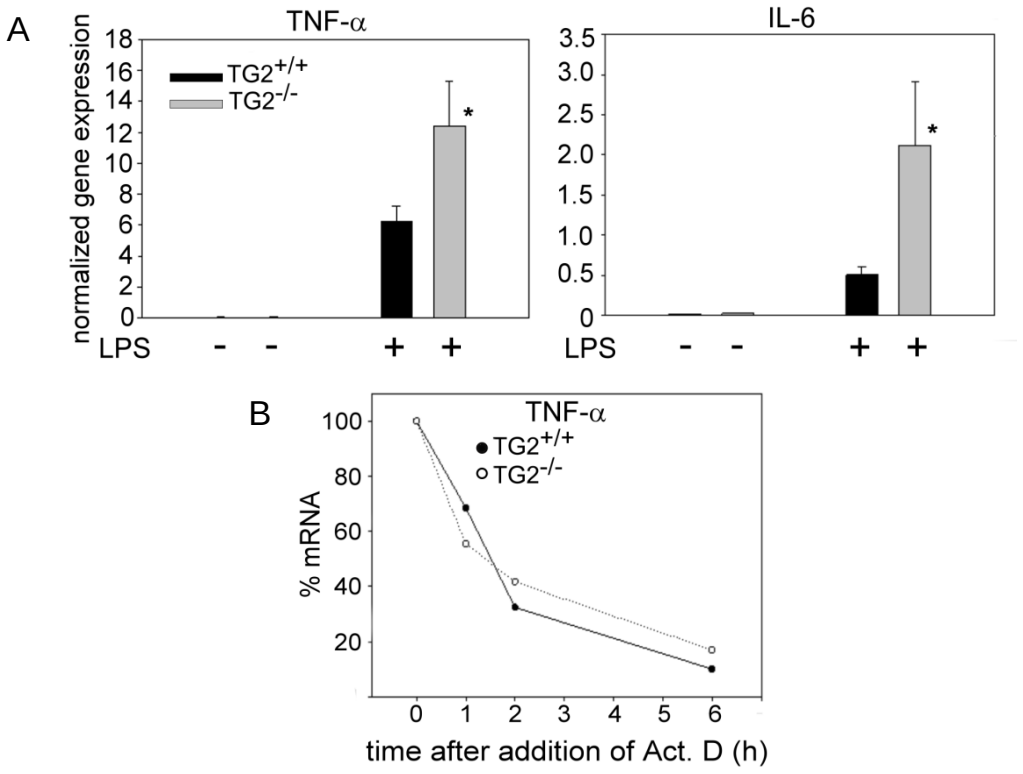


Figure 14. TG2 null macrophages respond to LPS stimulation by an enhanced NF-κB activation as compared to their wild-type counterparts, and this phenomenon is not related to an altered cell surface expression of CD14 or TLR4.

*(A) Quantitative RT-PCR analysis of TNF-α and IL6 mRNA expression in wild type and TG2 null peritoneal macrophages cultured for 1 h with or without 100 ng/ml LPS. The results are representative of three independent experiments and are shown as mean ± SD. (B) Measurement of TNF-α mRNA stability in wild-type and TG2 null peritoneal macrophages. Cell were treated with 100 ng/ml LPS for 1 h followed by addition of 1 μg/ml Actinomycin D. TNF-α mRNA was measured by quantitative RT-PCR. The results are representative of three independent experiments and are expressed as fold induction normalized to the wild-type control samples, and are shown as mean ± SD (*p<0.05).*

Since the signaling pathways induced by LPS transduce their effect on pro-inflammatory cytokine production partly via activating NF-κB, and these pathways regulate the degradation of the inhibitory subunit IκBα (Kawai and Akira, 2006), a negative regulator of NF-κB, we decided to determine the IκBα levels in wild type and TG2 null macrophages following LPS stimulation. As shown in Fig.15A, there was no change in the kinetics of the IκBα degrada-

tion induced by LPS stimulation, but the basal levels of IκBα in TG2 null macrophages were significantly lower than that of the wild type cells.

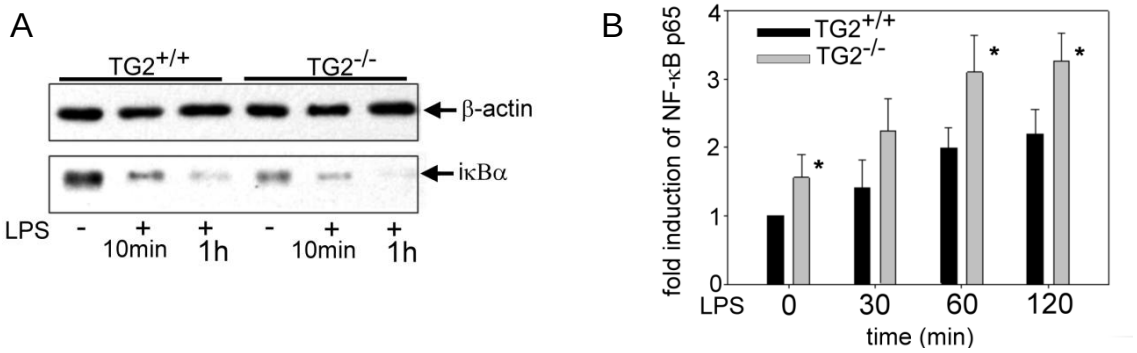


Figure 15. TG2 null macrophages respond to LPS stimulation by an enhanced NF-κB activation as compared to their wild-type counterparts, and this phenomenon is not related to an altered cell surface expression of CD14 or TLR4.

*(A) Western blot analysis of IκBα degradation in wild type and TG2 null macrophages after exposure to 100 ng/ml LPS. β-actin was used as a loading control. (B) Determination of the amounts of nuclear p65 NF-κB subunit in control and LPS-stimulated macrophages. Wild type and TG2 null peritoneal macrophages were treated with 100 ng/ml LPS for the indicated time periods. Nuclear p65 subunit was detected with TransAM p65 kit. The results are representative of three independent experiments and are expressed as fold induction normalized to the wild-type control samples, and are shown as mean ± SD (*p<0.05).*

IκBα is commonly associated with the NF-κB dimer p50 (NF-κB1)/p65 (RelA). Following proteolytic degradation of IκBα by the proteasome, the NF-κB dimer becomes free to enter the nucleus and to activate transcription of target genes. While NF-κB p65 is transcriptionally active, NF-κB p50 does not possess a transactivation domain (Baldwin 1996). Thus, though p65 and p50 can synergistically activate for example the TNF-α promoter (Liu *et al.*, 2000), the presence of p65 is crucial for the initiation of transcription (Baldwin 1996). That is why we decided to test the nuclear translocation of the p65 subunit of NF-κB by the TransAM p65 kit (ActiveMotif). As shown in Fig.32B, LPS stimulation induced the nuclear translocation of p65 in both types of macrophages. However, in accordance with the lower IκBα levels in TG2 null cells, both the basal and the LPS-induced levels of p65 were higher in the nucleus of TG2 null macrophages than in that of the wild-type macrophages at each time point tested. These data indicate that the loss of TG2 alters a signaling pathway that is coupled to the control of IκBα levels.

4.1.4. TG2 is required on the cell surface to decrease LPS-induced pro-inflammatory cytokine production in TG2 null macrophages

Previous studies have already shown association between NF-κB activation and TG2. In LPS-stimulated microglial cells, TG2 activates NF-κB via a novel pathway. Rather than stimulating phosphorylation and degradation of IκBα, TG2 interferes with its action by protein polymerization (Lee *et al.*, 2004). On the other hand, in cancer cells overexpression of TG2 enhances NF-κB activation by promoting integrin signaling (Verma and Mehta, 2007).

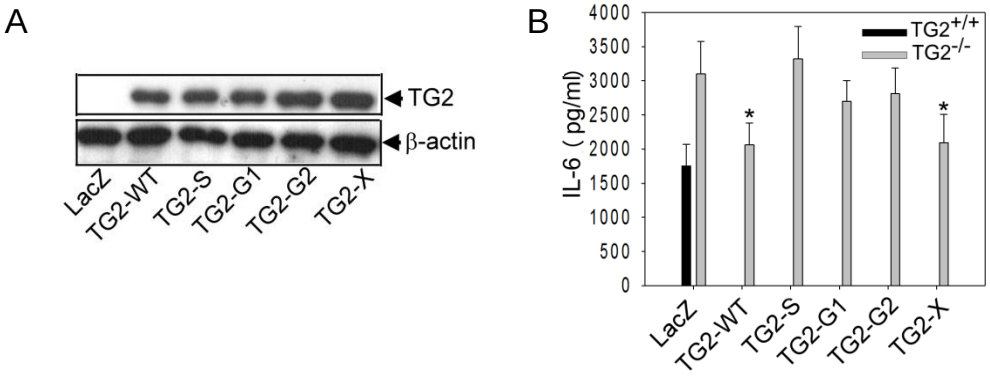


Figure 16. Integrin β_3-associated cell surface TG2 regulates pro-inflammatory cytokine production.

*(A) Western blot analysis showing TG2 expression in TG2 null peritoneal macrophages infected with adenoviruses carrying LacZ gene, wild-type, secretion deficient (TG2-S), guanine nucleotide binding deficient (TG2-G1 and -G2) or crosslinking function deficient (TG2-X) TG2 genes. (B) IL-6 production of wild type (black bar) or TG2 null (grey bars) peritoneal macrophages infected with the indicated constructs. Macrophages were stimulated with 100 ng/ml LPS for 1 h. After 1h incubation LPS was replaced with fresh medium. Supernatants were collected and frozen after 5h. IL-6 cytokine levels were determined by ELISA technique. Results are shown as mean ± SD of three independent experiments (*p<0.05).*

However, if these mechanisms exist in macrophages, loss of TG2 should lead to a decreased, not to an enhanced LPS signaling. To answer which biological functions of TG2 are required to downregulate LPS-induced pro-inflammatory cytokine production, adenoviral gene delivery system was used to transfect primary peritoneal macrophages with various mutants of TG2 (Fig.33A).

The following TG2 mutants were tested: a crosslinking activity mutant (TG2-X) by replacement of catalytic *Cys*^{277} by Ser (Lee *et al.,* 1993), two guanine nucleotide binding mutants by replacement of *Lys*^{172} and *Phe*^{173} by Asn and Asp (TG2-G1) (Iismaa *et al.*, 2000), and of *Glu*^{578}and *Arg*^{579} by Gln and Glu (TG2-G2) (Begg *et al.*, 2006), and a secretion mutant (TG2-S) by replacement of *Tyr*^{274} by Ala (Balklava *et al.,* 2002). As shown in Fig.33B, using IL-6 production as a read out, only the wild type and the crosslinking mutant were able to fully revert the LPS sensitive phenotype, while the secretion mutant and the two guanine nucleotide binding mutants were ineffective. Our data indicate that cell surface TG2 regulates negatively the LPS-induced pro-inflammatory cytokine production, and the crosslinking activity is not required for this effect. In addition, these data confirm those findings, which suggested that the enhanced LPS-induced pro-inflammatory cytokine production of TG2 null macrophages is not related to the lack of TGF-β activation, as it would require the crosslinking activity of TG2 (Nunes *et al.,* 1995).

4.1.5. Altered $\alpha_v\beta_3$ signaling is responsible for the enhanced LPS-induced pro-inflammatory cytokine production in macrophages

On the cell surface TG2 has been shown to act as an integrin-binding adhesion coreceptor and acting so to suppress Src kinase activity (Janiak *et al.,* 2006). Since it has been reported that $\alpha_v\beta_3$ integrin signaling can lead to NF-κB activation and enhance LPS-induced NF-κB signaling via activating Src kinase (Courter *et al.,* 2005; Lee *et al.,* 2007), we decided to test the potential role of an altered $\alpha_v\beta_3$ integrin signaling in the enhanced LPS-induced pro-inflammatory cytokine production of TG2 null macrophages. Preincubation of TG2 null macrophages with soluble vitronectin, an inhibitor of the $\alpha_v\beta_3$ integrin signaling (Preissner, 1991; Savill *et al.,* 1990), decreased the LPS-induced pro-inflammatory cytokine production on mRNA levels, indicating that $\alpha_v\beta_3$ integrin signaling promotes the LPS-induced pro-inflammatory cytokine production in TG2 null macrophages (Fig.34A). Interestingly, the same treatment enhanced the pro-inflammatory cytokine production by wild-type macrophages (Fig.34B). In line with these observations, LPS-induced IκBα levels were further decreased following vitronectin treatment in wild-type cells (Fig.34D), but remained more elevated in knock out cells (Fig.34C). Since cell surface TG2 was reported to crosslink soluble vitronectin (Sane *et al.,* 1988), and crosslinked soluble vitronectin might enhance instead of inhibiting $\alpha_v\beta_3$ integrin signaling, for wild-type macrophages we repeated the experiments in

the presence of R294, a non-permeable TG2 inhibitor, which blocks the crosslinking activity of cell surface TG2 (compound 4 in Griffin *et al.*, 2008).

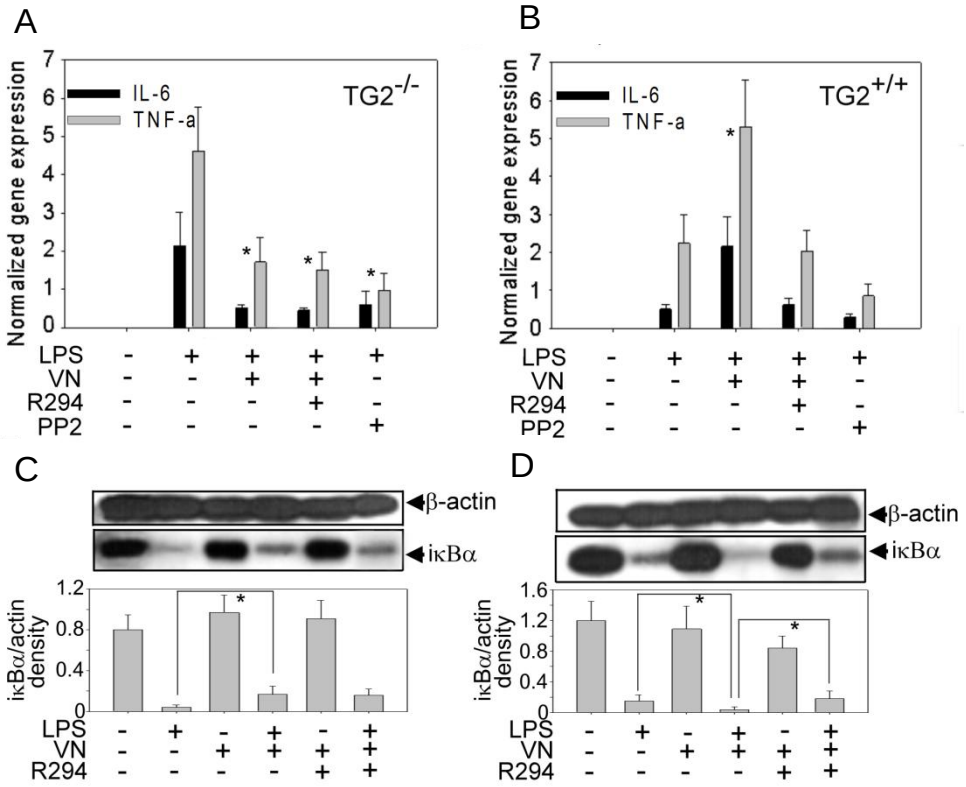


Figure 34. Integrin β_3-associated cell surface TG2 regulates pro-inflammatory cytokine production.

(A, B) Quantitative RT-PCR analysis of TNF-α *and IL6 mRNA expression in (A) TG2 null and (B) wild-type peritoneal macrophages cultured for 1 h with or without 100 ng/ml LPS alone or after one hour pretreatment of soluble vitronectin (VN)(1 μg/ml), 2 hrs R294 (100 μM) or 24 h pretreatment of PP2 (2 μM). Target gene expression was normalized to cyclophilin D. The results are representative of three independent experiments and are expressed as mean ± SD. (* significantly different from the corresponding LPS treated samples, p<0.05 determined by unpaired Student's t-test). (C, D) Western blot analysis of* IκBα *degradation in (C) TG2 null and (D) wild-type peritoneal macrophages following treatment by 100 ng/ml LPS alone or together with 1 μg/ml VN or 100 μM R294 (*p<0.05).*

As shown in Fig.34B, in the presence of the TG2 inhibitor and soluble vitronectin wild-type cells responded to LPS with nearly the same amount of cytokine mRNA expression as in the absence of them. In control experiments, addition of the TG2 inhibitor did not influence the

response of TG2 null cells to soluble vitronectin (Fig.34A). Alterations in the IκB levels mirrored these changes in the cytokine mRNA expression (Fig.34C and D). Altogether these data indicate that under our experimental conditions in wild type cells ligand-activated $\alpha_v\beta_3$ integrin signaling, which can be inhibited by soluble vitronectin, does not play a determining role in influencing LPS signaling (though stimulation of it by crosslinked vitonectin is capable of its enhancement), while in TG2 null cells it does.

4.1.6. In TG2 null macrophages enhanced $\alpha_v\beta_3$ integrin-induced src family tyrosine kinase activation is responsible for the enhanced NF-κB signalling

Next, we decided to test the activation state of Src family tyrosine kinases in TG2 null macrophages. The Src-family tyrosine kinases are highly conserved allosteric enzymes playing a key role in integrin cellular signaling. Phosphorylation of Tyr416 plays a central role in their activation (Banavali and Roux, 2009). In line with the report, which suggested that TG2 might negatively control $\alpha_v\beta_3$ integrin-regulated Src kinase activity (Janiak *et al.,* 2006), an enhanced phosphorylation of c-src family kinases was detected at Tyr 416 in TG2 null cells without a detectable change in the c-src protein levels as compared to the wild type cells (Fig.35A). LPS stimulation enhanced the amount of phosphorylated Src family tyrosine kinases in both types of macrophages (Fig.35A), in line with previous publications, which showed that Src kinase is also involved in LPS signaling (Lee *et al.,* 2007; Kang *et al.,* 2005).

The cytoplasmic domain of β_3 integrin contains tyrosines at positions 747 and 759 in domains that have been implicated in regulation of $\alpha_v\beta_3$ function and that serve as potential substrates for Src family kinases (Datta *et al.,* 2002). Phosphorylation of Tyr at residue 747 was reported to be required for optimal post-ligand binding effects (Schaffner-Reckinger *et al.,* 1998), as well as for proper binding of the integrin ligands (Datta *et al.,* 2002) thus participating in both in the "outside in" and "inside out" integrin signaling. To test the activation state of β_3 integrin in TG2 null cells both the level of β_3 integrin and the phosphorylation state of its Tyr747 residue were determined by Western blot analysis. As shown in Fig.35B, in accordance with our previous findings (Tóth *et al.,* 2009a; Tóth *et al.,* 2009b) the levels of β_3 integrin were elevated in TG2 null macrophages. While in wild-type cells β_3 integrin was only slightly phosphorylated at the Tyr747 site, in TG2 null cells the phosphorylation level was much more pronounced indicating an enhanced activation of integrin β_3 in the absence of TG2. However, phosphorylation of β_3 integrin and Src tyrosine kinases seems to be related to

each other in reciprocal way, as inhibition of $\alpha_v\beta_3$ integrin by soluble vitronectin decreased the phosphorylation level of Src tyrosine kinase, and similarly inhibition of Src tyrosine kinase with 2 μM PP2 [(4-amino-5-(4-chlorophenyl)-7-(***t***-butyl)pyrazolo[3,4-***d***]pyrimidine)], an Src tyrosine kinase family inhibitor, inhibited the phosphorylation state of $\alpha_v\beta_3$ integrin (Fig.35C).

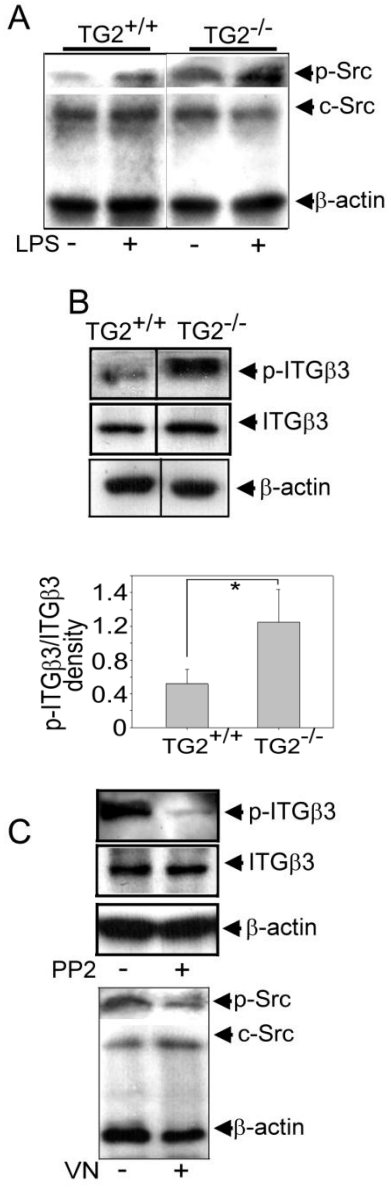


Figure 35. Loss of TG2 leads to enhanced $\alpha_v\beta_3$ integrin and Src kinase activity.

*(A) Increased Src family kinase phosphorylation in TG2 null macrophages. Western blot analysis of Tyr^{416} phosphorylation of Src family tyrosine kinase in resting wild type and TG2 null peritoneal macrophages and after 30 min LPS (100 ng/ml) exposure. (B) Increased basal integrin β_3 activity in TG2 null macrophages. Representative western blot analysis showing Tyr 474 phosphorylation of the integrin β_3 subunit in resting wild type and TG2 null peritoneal macrophages. β-actin was used as loading control. Densitometric quantification of phospho-integrin β_3 level normalized to total integrin β_3 levels in wild type and TG2 null macrophages (n=3, * statistically different from WT, p<0.05 determined by unpaired Student's t-test). (C) Effect of 1 hour soluble vitronectin (1 μg/ml), pretreatment on the Tyr416 phosphorylation of Src family tyrosine kinase, or 24 h PP2 (2 μM) pretreatment on the Tyr 474 phosphorylation of the integrin β_3 subunit in resting TG2 null peritoneal macrophages β-actin was used as loading control.*

Then we tested whether the decreased IκBα levels could be related to the enhanced activation of Src. Preincubation of macrophages with 2 μM PP2 for 24 hours did not affect their viability, but as shown in Fig.36, equalized the basal levels of IκBα detected in the wild-type and TG2 null macrophages indicating that the enhanced activity of Src kinase is responsible for the altered IκBα levels in TG2 null cells. In addition, inhibition of Src kinase delayed LPS-induced degradation of IκBα in both types of cells, and in the presence of PP2, no difference

was found in the IκBα levels following addition of LPS. Finally, addition of PP2 decreased, but at the same equalized the LPS-induced mRNA production of the two pro-inflammatory cytokines within the wild type and TG2 null macrophages (Fig.36A and B). All together, these data provide evidence for the involvement of the $\alpha_v\beta_3$ integrin regulated Src family tyrosine kinases in the altered LPS signaling in TG2 null macrophages.

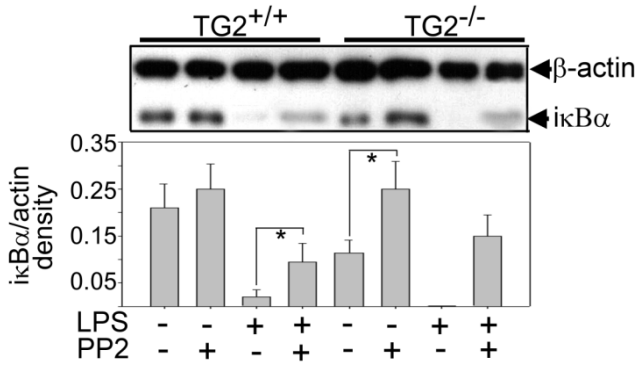


Figure 36. The inhibition of Src kinase activity equalizes the IκBα levels of wild type and TG2 null macrophages.

*Western blot analysis of IκBα degradation in wild-type and TG2 null peritoneal macrophages with or without a 24 h PP2 (2 μM) pretreatment after triggering or not with 100 ng/ml LPS for 30 min. β-actin was used as loading control (n=3; *p<0.05).*

4.2. INVOLVEMENT OF ADENOSINE A2A RECEPTORS IN APOPTOTIC CELL INDUCED SUPPRESSION OF INFLAMMATION

4.2.1. Apoptotic cell uptake leads to adenosine release and increased gene and cell surface expression of $A_{2A}R$ in macrophages

At the beginning of our study, the first and most important point was the determination of possible ADO production by macrophages during the clearance of ACs. ADO was found in the culture medium of macrophages exposed to ACs (Fig. 10). The release of ADO was not specific for thymocytes, because the uptake of As_2O_3-treated apoptotic NB4 acute promyelocytic leukemia cells (Chen *et al.*, 1997) also triggered ADO release. In parallel, the ADO concentration in the culture medium alone or in the culture medium of macrophages or thymocytes cultured alsone was below the detection limit. The amount of ADO produced by macrophages engulfing ACs was in the range of the $A_{2A}R$ receptor affinity range. When we blocked the phagocytosis with actin polymerization inhibitor cytochalasin D, the ADO disappeared from cell culture media, suggesting that ADO production of AC-treated macrophages is engulfment-dependent.

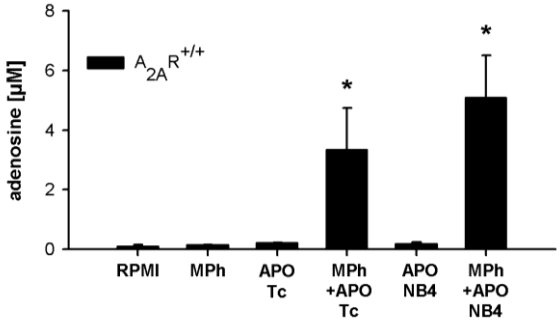


Figure 10. Macrophages exposed to apoptotic cells release adenosine.

*Wild type peritoneal macrophages (MPh) were incubated alone or in the presence of apoptotic thymocytes (APO) or NB4 cells in 1:10 ratio. After 2 hrs. of phagocytosis the supernatants were replaced with fresh culture media. After 5 hrs. of incubation (at 37°C) the supernatants were collected, deproteinized with 5 ml ice cold 0.6 N $HClO_4$ and stored on -80°C. The determination of ADO concentration was carried out with a reverse phase HPLC method. Results are expressed as mean ± S.D. of three independent experiments (*p<0.05).*

Moreover, AC exposure not only triggered ADO release in macrophages, but also enhanced the cell surface level of $A_{2A}R$ in time-dependent manner (Fig.11). We made four important

observations in relation to $A_{2A}R$ upregulation. First, AC-treatment modulates the expression of $A_{2A}R$ on transcriptional level, because the induction disappeared in the presence of transcription inhibitor actinomycin D, or protein synthesis inhibitor cycloheximide (Fig. 11B).

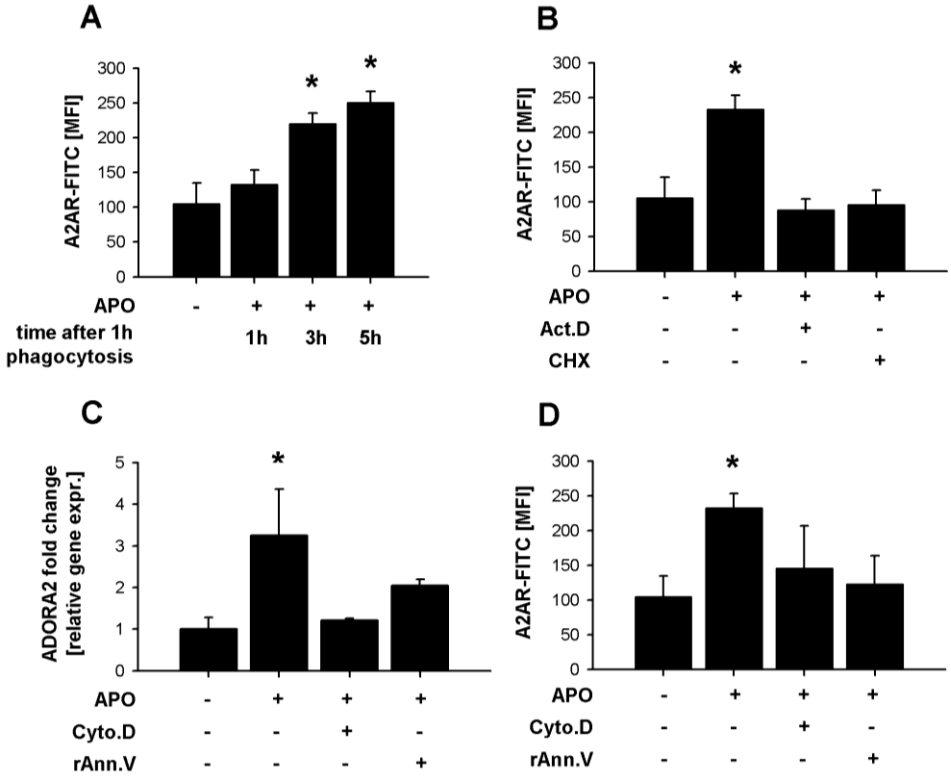


Figure 11. Apoptotic cell uptake leads to *de novo* adenosine $A_{2A}R$ synthesis in peritoneal macrophages.

(A) Wild-type peritoneal macrophages (MPh) were exposed for one hr. to apoptotic thymocytes (APO) alone or (B) in combination with 0.1 μg/ml cycloheximide (CHX) or 10 μM actinomycin D (Act.D). Thymocytes were then washed away and macrophages were further incubated (A) for the indicated time periods or for (B) 3 hrs. Cell surface expression of $A_{2A}R$ was determined by flow cytometry. (C-D) Blocking of apoptotic cell phagocytosis abolishes the increase of $A_{2A}R$ level. Phagocytosis was inhibited by pretreating macrophages with 50 mM cytochalasin D (Cyto.D) or by masking the phosphatidylserine on the apoptotic cell surface with recombinant annexin V (rAnn.V; m10 μg/10^5 apoptotic cell). After one hr. co-incubation apoptotic cell were washed away and macrophages were cultured for further 2 hrs (C) or 3 hrs (D). $A_{2A}R$ level was determined by quantitative PCR (C) or by flow cytometry (D). MFI: mean fluorescence intensity. Results are expressed as mean ± S.D. of three or four independent experiments ($p<0.05$).*

Indeed, induction on the level of mRNA was evident following the engulfment of ACs (Fig.11C). Secondly, when we prevented the uptake of ACs with cytochalasin D pretreatment, or blocked the PS-mediated recognition process by masking the surface of ACs with recombinant annexin V (which binds to PS; Hoffmann *et al.,* 2001) the $A_{2A}R$ inducing effect of ACs was inhibited (Fig.11C and D). These findings demonstrate that engulfment of ACs, rather than their recognition triggers enhanced $A_{2A}R$ expression. Third, the induction in $A_{2A}R$ expression during phagocytosis is specific for the engulfment of ACs, as uptake of neither necrotic nor antibody-coated cells triggered it (Fig.12).

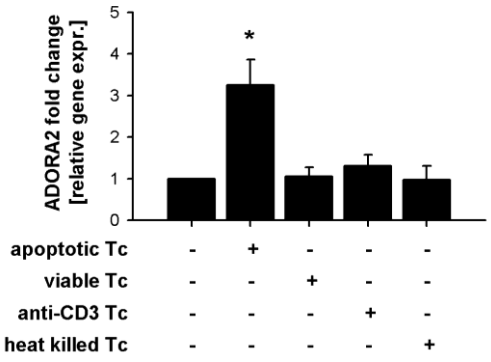


Figure 12. The adenosine $A_{2A}R$ inducing effect is specific for apoptotic cell uptake.

*Wild type peritoneal macrophages were coincubated with various target cell types: apoptotic, living, heat killed (45 min, 55°C) or anti-CD3-pretreated (10 μg/ml, 20 min) thymocytes for 1 hr in 1:10 ratio. After washing away the apoptotic cells and replacing media, mRNA was collected 2 hours later. Results are expressed as mean ± S.D. of four independent experiments (*p<0.05).*

Finally, we tested the possible involvement of two lipid-sensing nuclear receptors (LXR and PPARδ) in the $A_{2A}R$ upregulating effect of ACs. Both 22-(R)OH-cholesterol, an LXR agonist, and GW501516, a PPARδ agonist, promoted the mRNA expression of $A_{2A}R$ in peritoneal macrophages (Fig.13 A and B) indicating that LXR and PPARδ might mediate the effect of AC engulfment on $A_{2A}R$ expression. Since the effect of these agonists might be not be fully specific, to prove further the involvement of these receptors in the $A_{2A}R$ upregulation LXR knock out and PPARδ knock out macrophages were also tested for their response. While in case of PPARδ$^{-/-}$ macrophages the upregulation of $A_{2A}R$ was attenuated as compared to their wild-type controls (Fig.13), we could not draw a definite conclusion from the LXR KO mice, as their wild type control did not show an upregulation (Fig.13D). We have no explanation why induction of the expression of $A_{2A}R$ was seen in macrophages on FVB and 129/SvJ backgrounds, but was not seen in mice on a mixed background of C57Bl/6 and 129Sv. These data imply that lipid-sensing receptors might mediate the effect of AC on the $A_{2A}Rs$, but since

the biological activity of these receptors overlap, only testing double knock out cells would give a full answer.

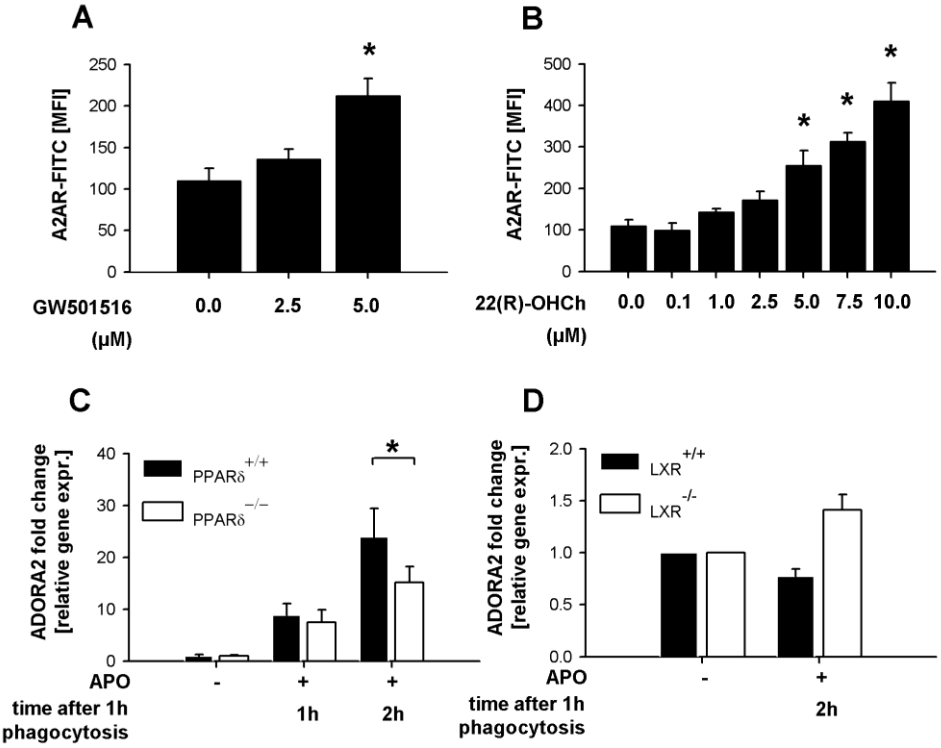


Figure 13. LXR and PPARδ might mediate the effect of apoptotic cell engulfment on $A_{2A}R$ expression.

*Macrophages were treated with GW501516, a PPRδ agonist (A), or by 22-(R)OH-cholesterol, an LXR agonist (B), for 3 hrs. Cell surface expression of $A_{2A}R$ was determined by flow cytometry. ADORA2 gene expression level was also determined in PPARδ knock bone marrow derived (C) and LXR null peritoneal macrophages (D). Macrophages were coincubated with apoptotic thymocytes (APO) for 1 hr. Apoptotic cells were then washed away and macrophages were further incubated for 2 hours. The gene expression levels were measured by quantitative PCR. MFI: mean fluorescence intensity. Results are expressed as mean ± S.D. of three or four independent experiments (*p<0.05)*

4.2.2. Loss of adenosine $A_{2A}R$ influences the pro-inflammatory cytokine production of macrophages engulfing apoptotic cells

In the next step we studied the effect of $A_{2A}R$-deficiency on the cytokine profile of resting and AC-treated peritoneal macrophages using a highly sensitive cytokine antibody array method, enabling the simultaneous detection of low concentrations of multiple cytokines in

one assay (picogram per milliliter range). The map of the 40 cytokines detected on the membranes is diagrammed in Table 2 (see Materials and Methods).

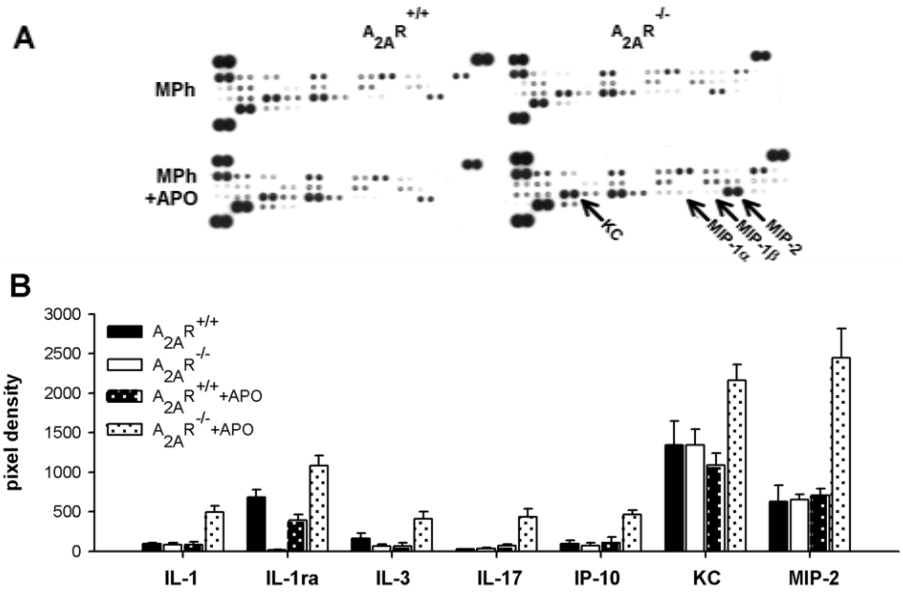


Figure 14. Adenosine $A_{2A}R$-deficient macrophages respond to apoptotic cells with enhanced chemoattractant production.

(A) Cytokine panel of control and apoptotic cell (APO) treated wild-type and $A_{2A}R$ null peritoneal macrophages. Peritoneal macrophages were co-incubated with apoptotic thymocytes for one hr. (MPh:APO=1:10) followed by removal of apoptotic cells and addition of fresh medium. Supernatants were collected five hours later and cytokine levels were determined by cytokine array. Arrows highlight neutrophil chemoattractants, which are significantly overproduced by $A_{2A}R$ null macrophages. (C) Cytokines, which levels were significantly different ($p<0.05$) in the supernatants analyzed by cytokine array. The density of MIP-1α and β were low to show together with the other cytokines. Results are expressed as mean ± S.D. of three independent experiments.

The results reported in Fig.14A show that 85% of all available cytokines on the filters were detectable, even though some were at a very low level. The loss of the $A_{2A}R$ did not affect significantly the cytokine profile of resting macrophages. In contrast, we found nine cytokines in AC-treated samples whose levels were increased in the lack of $A_{2A}R$. These cytokines include IP-10, KC, and MIP-2, which act as chemoattractants for neutrophils and/or other cell types (Neville *et al.*, 1998; Iida and Grotendorst, 1990; Wolpe *et al.*, 1989; Wolpe and Cerami, 1989). The pro-inflammatory cytokines IL-17 (Kolls and Lindén, 2004) and IL-1α and

IL-3, which stimulates the differentiation of multipotent hematopoietic stem cells into the myeloid direction and proliferation of all cells in the myeloid lineage (Yang *et al.*, 1986), as well as the anti-inflammatory cytokine IL-1ra also, were produced in an enhanced amount. In addition, release of MIP-1α, and -1β were increased also. Since wild type and $A_{2A}R$ null macrophages have similar phagocytotic activity and capacity (Fig.15), the detected alterations are not consequences of phagocytosis-deficiency of cells lacking $A_{2A}R$.

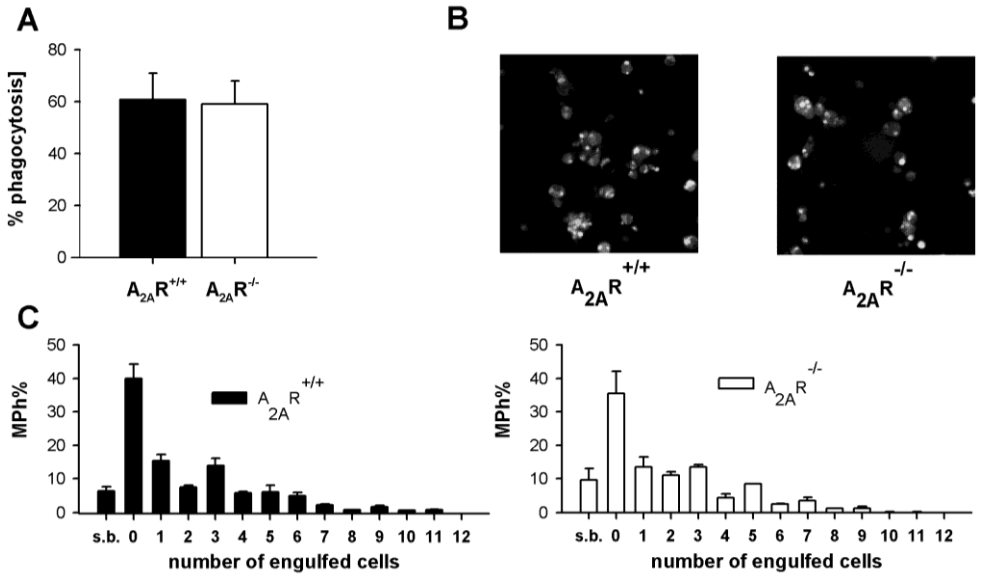


Figure 15. The altered cytokine profile of $A_{2A}R$ null macrophages is not due to phagocytotic deficiency.

(A) Flow cytometric measurement of capacity of wild type and A2A null peritoneal macrophages to take up apoptotic thymocytes. (B-C) The number of engulfed fluorescently labeled apoptotic cells within WT or A2AR-null macrophages counted by confocal microscopy following 1h of phagocytosis. "s.b." indicates surface bound cells. Results are expressed as mean ± S.D. of three independent experiments.

Among the nine cytokines overproduced by $A_{2A}R^{-/-}$ macrophages, MIP-2 showed the most dynamic change in response to AC exposure, and MIP-2 and KC levels were detected in the highest amounts (Fig.16). No cytokines were detected in the supernatants when thymocytes (viable or apoptotic) were incubated alone (data not shown), demonstrating that the secreted cytokines were macrophage-related.

To exclude the possibility, that alterations in the cytokine profile are a result of $A_{2A}R$-related developmental effects in the knock out cells, we repeated the cytokine profiling experimt in

the presence of highly specific $A_{2A}R$ specific antagonist, SCH442416, and the highly specific $A_{2A}R$ agonist CGS21680. The way as the selective concentration of these compounds was determined is described in the Fig.9 (see Material and Methods).

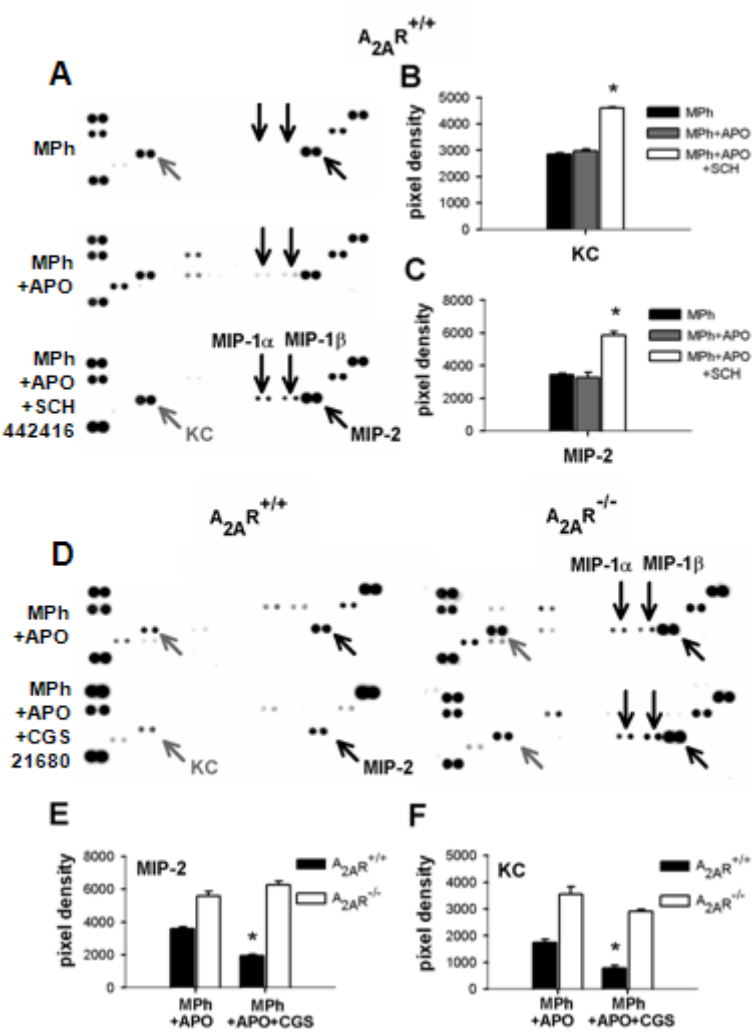


Figure 16. As compared to wild type controls, MIP-2 and KC production is enhanced by both $A_{2A}R$ null or $A_{2A}R$ antagonist treated wild-type macrophages while engulfing apoptotic cells.

*(A) Cytokine panel of control, apoptotic cell (APO) exposed and SCH442416 ($A_{2A}R$-specific antagonist) treated wild-type macrophages (MPh). Peritoneal macrophages were preincubated with 10 nM SCH442416 for 30 min, then they were exposed to apoptotic thymocytes for one hr (MPh:APO=1:10). 5 hours after the removal of apoptotic cells supernatants were collected and cytokine levels were determined by cytokine array. Arrows highlight KC (B), MIP-2 (C), overproduced by SCH442416 treated macrophages. (D) Cytokine panel of control, apoptotic cell exposed and CGS21680 ($A_{2A}R$-specific agonist) treated wild type and $A_{2A}R$ null macrophages. Peritoneal macrophages were pre-incubated with 10 nM CGS21680 for 30 min, then they were exposed to apoptotic thymocytes for one hr (MPh:APO=1:10). 5 hours after the removal of apoptotic cells supernatants were collected and cytokine levels were determined by cytokine array. Arrows highlight MIP-2 (E), KC (F) down regulated by CGS21680 in wild type, but not in $A_{2A}R$ null macrophages. Results are expressed as mean ± S.D. of three independent experiments (*p<0.05).*

As shown in Fig.16, using wild type macrophages we could confirm the enhanced expression of KC and MIP-2 in the presence of an $A_{2A}R$ antagonist as well, indicating that the altered pattern of KC and MIP-2 secretion observed in $A_{2A}R^{-/-}$ macrophages is indeed a consequence of the lack of the $A_{2A}R$ signaling during phagocytosis of ACs. In addition, the enhanced expression of MIP-1α β two further neutrophil chemoattractants (Wolpe and Cerami, 1989), was more clearly seen. Administration of CGS 21680 on the other hand, further decreased the amount of KC and MIP-2 produced by wild-type macrophages engulfing ACs, while had no significant effect on that by $A_{2A}R$ null macrophages engulfing ACs (Fig.16D, E, F).

Since the cytokine array is a semi-quantitative method, we confirmed the results by the usage of specific MIP-2 and KC ELISA. In harmony with the array results, the protein levels of both chemoattractants were highly increased in the conditioned media of AC treated A2AR null macrophages compared to the wild type ones (Fig.17A and B).

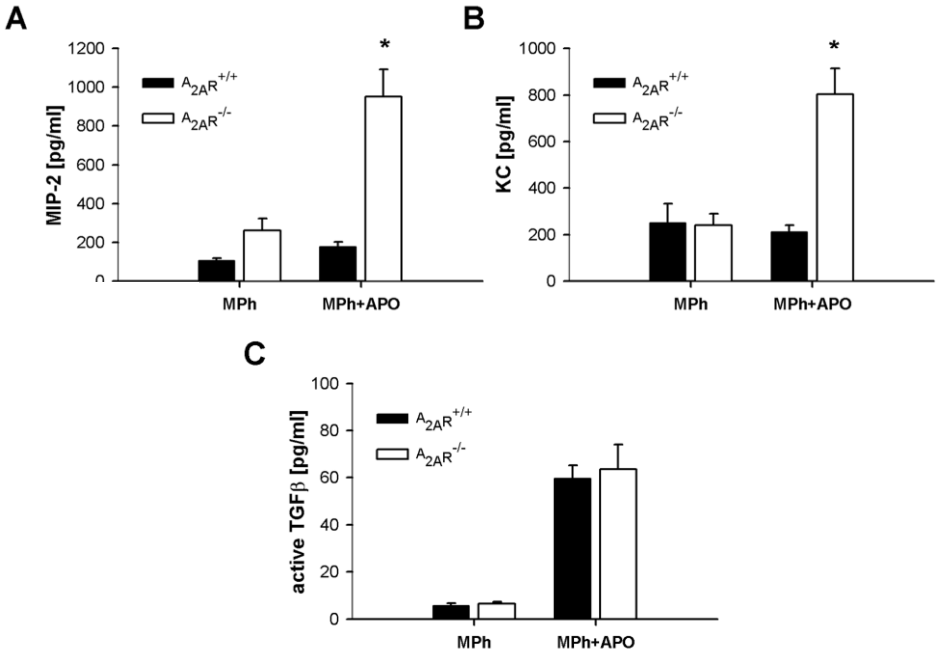


Figure 17. Cytokine production of wild type and $A_{2A}R$ null mice during phagocytosis of apoptotic cells.

*Wild-type and $A_{2A}R$ null macrophages (MPh) were exposed to apoptotic thymocytes (APO) for one hr (MPh:APO=1:10). 5 hours after the removal of apoptotic cells supernatants were collected. The levels of (A) MIP-2, (B) KC and (C) active TGF-β production were determined by ELISA. Results are expressed as mean ± S.D. of three independent experiments (*p<0.05).*

Previous studies indicated that in long-term (one-day) experiments, TGF-β and IL-10 might mediate the anti-inflammatory effects of ACs (Voll *et al.*, 1997; Fadok *et al.*, 1998; McDonald *et al.*, 1999), we checked whether TGF-β or IL-10 release is altered in macrophages lacking $A_{2A}R$. However, in such short-term experiments we could not detect the release of IL-10 neither with the cytokine array nor by the ELISA technique. Active TGF-β was detectable, but its production was not altered in the $A_{2A}R^{-/-}$ macrophages (Fig.17C). These data indicate that not an altered IL-10 or TGF-β production regulates the altered MIP-2 production in $A_{2A}R^{-/-}$ macrophages.

4.2.3. Macrophages not expressing adenosine $A_{2A}R$ induce migration of neutrophils when exposed to apoptotic cells in a sterile peritonitis model

To prove the biological significance of enhanced neutrophil chemoattractant production of $A_{2A}R$ null macrophages uptaking ACs, we decided to investigate whether injection of ACs affects the migration of neutrophils in $A_{2A}R^{-/-}$ mice. For this purpose, we used a sterile peritonitis model, in which we injected mice intraperitoneally, first with thioglycollate and then, 4 days later, with 2×10^6 ACs. As shown in Fig.18A, injection of apoptotic thymocytes did not induce a significant neutrophil migration into the peritoneum in wild-type mice, while in the $A_{2A}R^{-/-}$ mice a significant neutrophil migration was detected in this model. This was accompanied by enhanced levels of MIP-2 and KC in the peritoneal fluid of $A_{2A}R^{-/-}$ mice (Fig.18B). To confirm, that the enhanced KC and MIP-2 production is responsible for the phenomenon, blocking antibodies - anti-KC (50 μg) and anti–MIP-2 (50 μg) - or their isotype controls were injected together with the ACs. As shown in Fig.18C, addition of blocking antibodies to both KC and MIP-2 completely prevented the migration of neutrophils, while their isotype controls had no effect. Addition of blocking antibodies to MIP-2 alone did not fully block the migration of neutrophils. These data indicate that the loss of $A_{2A}R$ leads to sufficient neutrophil chemoattractant production by macrophages engulfing ACs to affect migration of neutrophils in an *in vivo* peritonitis model.

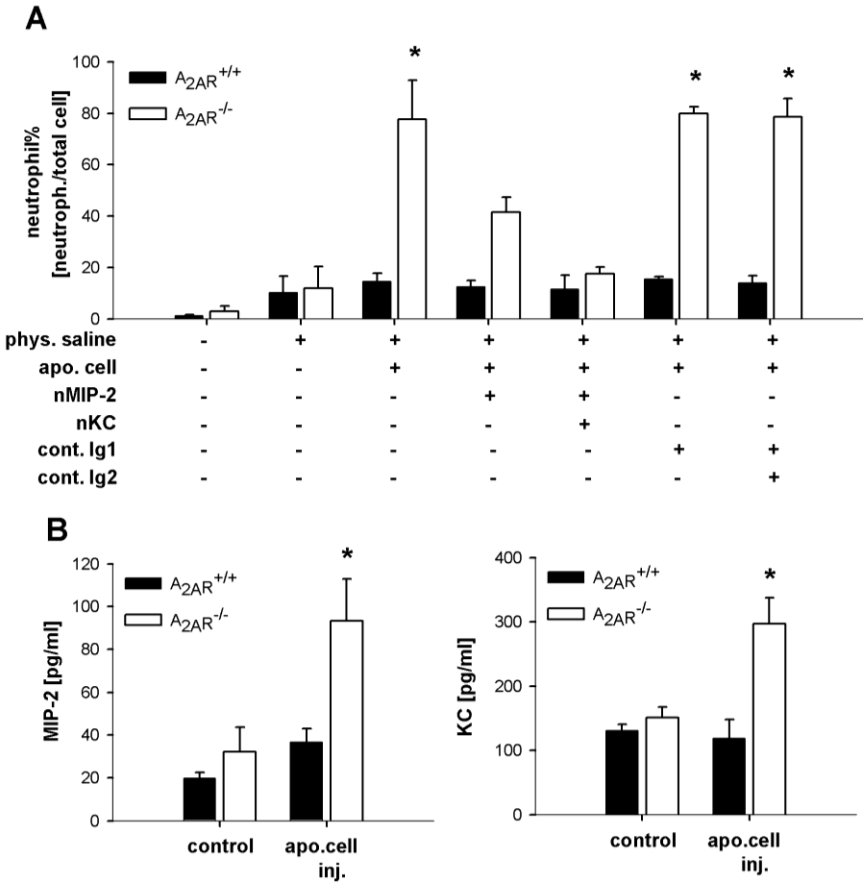


Figure 18. Increased MIP-2 production is accompanied with enhanced neutrophil migration *in vivo*.

*(A) Wild-type and $A_{2A}R$ null mice with sterile peritonitis were intraperitoneally injected with apoptotic cells (10^5 cells/mouse in physiological saline) or vehicle alone. Untreated control mice were uninjected. In some cases neutralizing MIP-2 and neutralizing KC antibodies (nMIP-2 and nKC abs) or their isotype controls were also injected. Three hrs. later peritoneal cells were collected and analyzed by flow cytometry for Gr-1-positivity. The levels of MIP-2 (B) and KC (C) in the lavage fluids were determined by ELISA at the same time point. Results are expressed as mean ± S.D. of five independent experiments (*p<0.05).*

4.2.4. Production of MIP-2 by $A_{2A}R^{-/-}$ macrophages exposed to apoptotic cells is related to lack of protein kinase A-mediated inhibition of NO production

Since MIP-2 showed the most dramatic changes during phagocytosis of ACs in $A_{2A}R$ null macrophages, we decided to investigate further the alterations in the regulation of this cytokine. First, we tested the role of the adenylate cyclase pathway, since many of the anti-

inflammatory effects of the $A_{2A}R$ were reported to be mediated via this signaling pathway (Haskó and Cronstein, 2004).

On one hand, we mimicked the action of ADO-$A_{2A}R$ system in $A_{2A}R$ null macrophages by the elevation of cAMP levels with cholera toxin; by the activation of adenylate cyclase with forskolin (Cassel and Selinger, 1977); or by the addition of a membrane permeable cAMP analogue dibutyryl-cAMP. Preincubation of $A_{2A}R^{-/-}$ macrophages with these compounds for 30 min. prevented the increase in MIP-2 levels when exposed to ACs (Fig.19A).

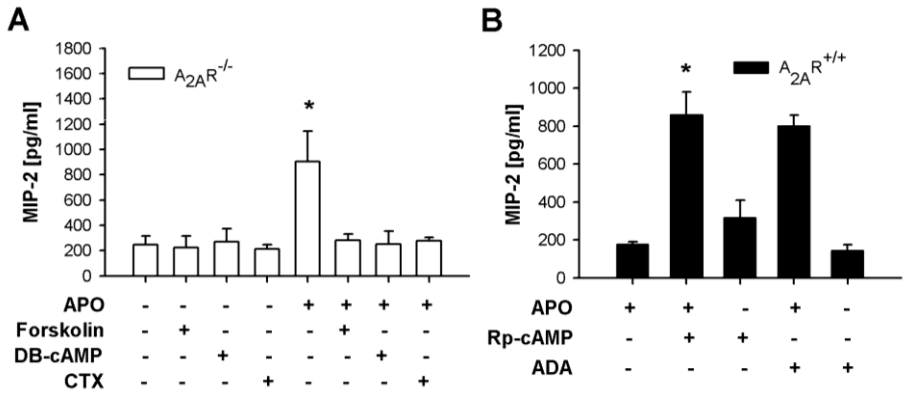


Figure 19. Transcriptionally-induced MIP-2 production by macrophages exposed to apoptotic cells is actively suppressed by the adenosine $A_{2A}R$ stimulated by adenosine in an autocrine way using the adenylate cyclase/protein kinase A signaling pathway.

*(A) $A_{2A}R$ null peritoneal macrophages were exposed to apoptotic cells alone or after a 30 min. pretreatment with forskolin (10 μM), cholera toxin (CTX; 100 ng / ml) or dibutyryl-cAMP (DB-cAMP; 100 μM). (B) Wild-type peritoneal macrophages were exposed to apoptotic cells alone or in combination with 100 μM Rp-cAMP triethylamine or adenosine deaminase (ADA; 1 U/ml). In all these experiments (A-B) apoptotic cells were washed away after one hr. and supernatants were collected after 5 hours to determine MIP-2 production by ELISA. Results are expressed as mean ± S.D. of five independent experiments (*p<0.05).*

On the other hand, the inhibition of PKA-mediated pathway by the usage of non-specific PKA inhibitor Rp-cAMPS triethylamine (van Haastert *et al.,* 1984), resulted in increased MIP-2 production in wild-type macrophages when they were exposed to ACs (Fig.19B). In addition, we observed the same effect when we eliminated the ADO from cell culture media by adenosine deaminase (ADA) treatment. Overall, these data indicate that MIP-2 production of macrophages exposed to ACs is actively suppressed by the $A_{2A}R$ stimulated by ADO in an autocrine way using the adenylate cyclase/PKA signaling pathway.

Inhibiting the *de novo* MIP-2 production by pretreatment with actinomycin D, or cycloheximide we were able to prevent the MIP-2-inducing effect of ACs, which suggests that the regulation occurs at the transcriptional level (Fig.20A). Indeed, engulfment of ACs induced the mRNA levels of MIP-2 in $A_{2A}R^{-/-}$ macrophages, but not in their wild-type ones (Fig.20B).

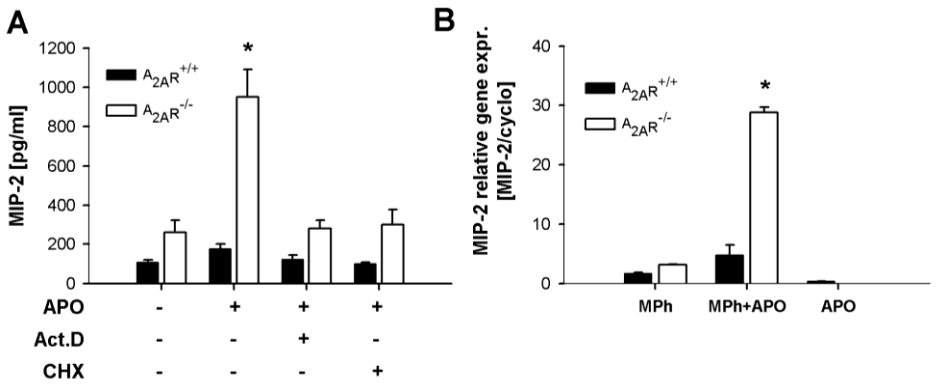


Figure 20. Apoptotic cells modulate MIP-2 production of macrophages on transcriptional level.

*(A) Wild type or $A_{2A}R^{-/-}$ peritoneal macrophages (MPh) were exposed to apoptotic cells (APO) alone or after a 30 min. pretreatment with 5 μg/ml actinomycin D (Act.D) or 0.1 μg/ml cycloheximide (CHX). Apoptotic cells were washed away after one hr. and supernatants were collected after 5 hours to determine MIP-2 production by ELISA. (B) Quantitative RT-PCR analysis of MIP-2 mRNA expression in wild-type resting, one hr. apoptotic cell exposed peritoneal macrophages at 2 hours after the removal of the apoptotic cells or apoptotic thymocytes. Expression values are represented as mean amount of target mRNA normalized to the expression of cyclophilin. Results are expressed as mean ± S.D. of five independent experiments (*p<0.05).*

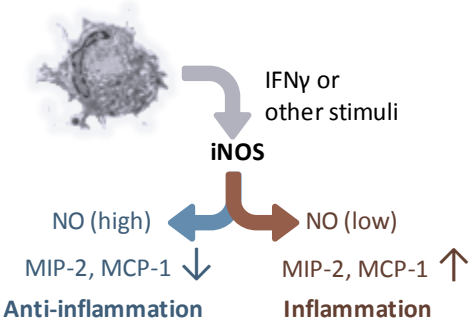


Figure 21. The immunoregulatory effect of NO. *(Kobayashi Y, 2010)*

According to previous studies AC-exposure triggers NO-release in macrophages (Shibata *et al.*, 2006), which can affect the MIP-2 production (Walpen *et al.*, 2001; Skidgel *et al.*, 2002). An interesting aspect of the biochemistry of NO is that both concentration and location are key determinants of its ability to activate different cell signaling pathways (Hill *et al.*, 2010).

We investigated the potential role of NO in three different ways. On one hand macrophages were exposed to nitric oxide synthase (NOS) inhibitor L-(G)-Nitro-L-arginine methyl ester (L-NAME) before the addition of ACs. The inhibition of NO production resulted in decreased MIP-2 protein (Fig.22A) and mRNA (Fig.22B) expression in both wild type and $A_{2A}R^{-/-}$ macrophages, indicating that NO production contributes to the effect.

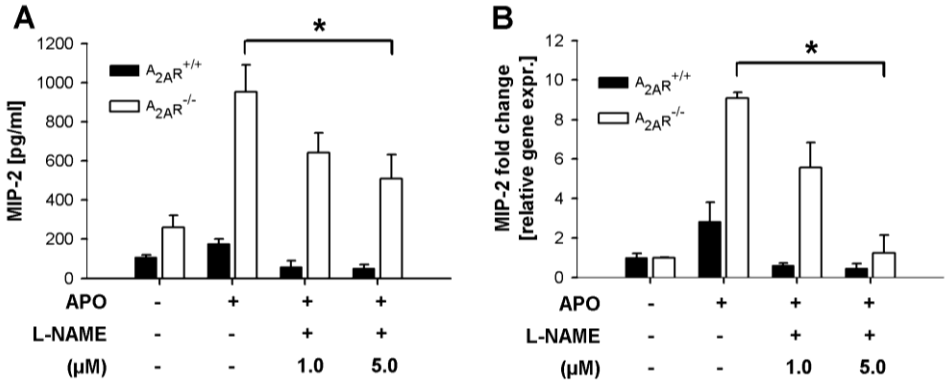


Figure 22. Apoptotic cell-induced MIP-2 production by adenosine $A_{2A}R$ null macrophages requires NO production.

*L-NAME, a nitric oxide synthetase inhibitor, prevents apoptotic cell-induced MIP-2 production by macrophages on both protein (A) and (B) mRNA level in a dose dependent manner. Wild type or $A_{2A}R$ null peritoneal macrophages were exposed to apoptotic cells (APO) for one hr. alone or after a 30 min. pretreatment with L-NAME in the indicated concentrations. After one hr. apoptotic cells were washed away and mRNA levels were determined 2 hrs later, while cytokine levels in the supernatant 5 hours later, L-NAME being constantly present. mRNA levels are expressed as fold changes as compared to the resting macrophages. Results are expressed as mean ± S.D. of five independent experiments (*p<0.05).*

On the other hand, we determined the NO release of wild type and $A_{2A}R$ null macrophages. As shown in Fig.23A the AC-induced NO production is significantly higher in $A_{2A}R$ null macrophages, than in the wild-type ones. Finally, we investigated the effect of exogenously added NO on MIP-2 production of AC-treated macrophages. The results are in line with our previously shown data: the addition of sodium nitropusside (SNP), a potent NO donor, enhanced AC-induced MIP-2 production in wild type macrophages (Fig.23B).

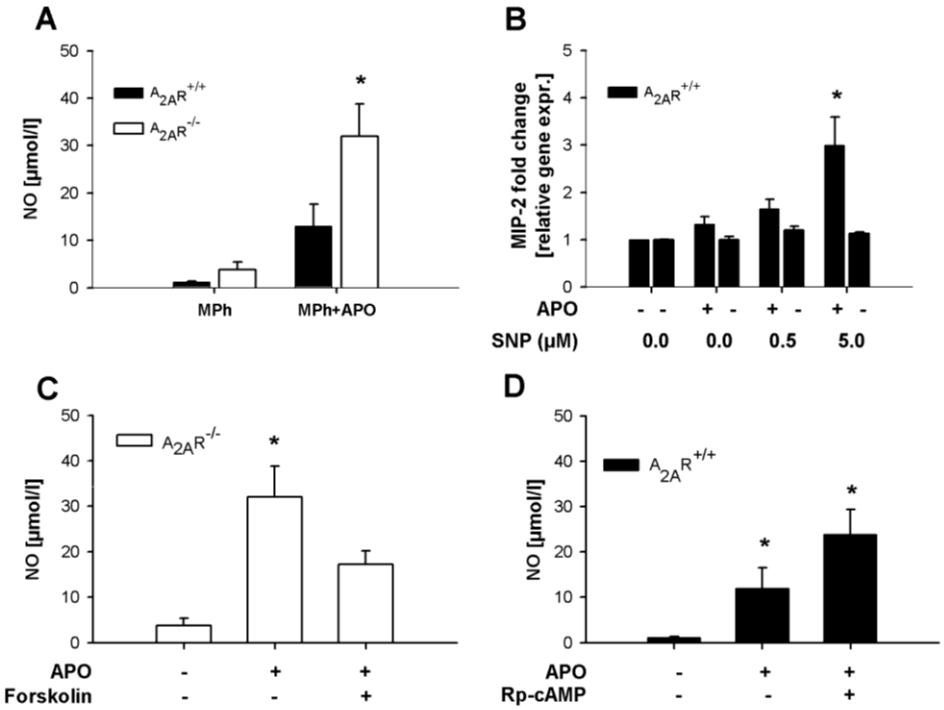


Figure 23. Overproduction of NO by $A_{2A}R$ null macrophages exposed to apoptotic cells is related to lack of PKA-mediated control.

(A) NO production of macrophages (MPh) exposed to apoptotic cells (APO) is enhanced in the absence of $A_{2A}R$. *Macrophages were exposed to apoptotic cells for one hr. NO production was determined following an additional hour. (B) The NO donor sodium nitropusside (SNP) enhances MIP-2 production, but only in macrophages exposed to apoptotic cells. Wild type peritoneal macrophages were exposed to the indicated concentrations of sodium nitropusside in the presence or absence of apoptotic cells. Apoptotic cells were washed away after one hr. MIP-2 mRNA levels were determined 2 hours later. (C) Forskolin, an adenylate cyclase activator, decreases NO production by* $A_{2A}R^{-/-}$ *macrophages. by* $A_{2A}R^{-/-}$ *peritoneal macrophages were exposed to apoptotic cells for one hr. alone or after a 30 min. pretreatment with forskolin (10 µM). Apoptotic cells were washed away and NO production was determined following an additional hr. (D) Inhibition of protein kinase A enhances NO production of wild-type macrophages exposed to apoptotic cells. Wild-type peritoneal macrophages were exposed to apoptotic cells for one hr. alone or after a 30 min. pretreatment with 100µM Rp-cAMP triethylamine. Apoptotic cells were washed away and NO production was determined following an additional hr. Results are expressed as mean ± S.D. of five independent experiments (*p<0.05).*

Taking together, our data indicate that NO contributes to the AC-induced MIP-2 production. However, addition of sodium nitropusside to macrophages alone was not able to induce MIP-2 production, implying that AC-derived signals contribute to the induction of MIP-2 (Fig.23B).

Similar to the induction of MIP-2 (Fig.19A and B), production of NO by $A_{2A}R^{-/-}$ macrophages engulfing ACs was inhibited by the adenylate cyclase activator forskolin (Fig.23C), while it was enhanced in wild type macrophages by the protein kinase A inhibitor Rp-cAMP triethylamine (Fig.23D) suggesting that $A_{2A}R$-mediated adenylate cyclase signaling inhibits primarily NO production.

4.2.5. Enhanced NO production in $A_{2A}R^{-/-}$ macrophages is accompanied by altered expressions of iNOS and arginase II

NO is synthesized intracellularly through the action of NOS enzymes, which catalyze the oxidation of L-arginine to L-citrulline and NO. *Endothelial NOS (eNOS)* generates the lowest levels of NO and it is found in neurons, epithelial cells, and cardiomyocytes. Its activity is controlled by Ca^{2+} and calmodulin, and post-translational modifications (Hill *et al.*, 2010). *Neuronal NOS (nNOS)* is constitutively present in neurons, skeletal muscle, and epithelial cells. It is also a Ca^{2+}/calmodulin-dependent form (Hill *et al.*, 2010). *Inducible NOS (iNOS)* has the highest capacity to generate NO. This isoform is expressed in multiple cell types in response to inflammatory stimuli.

The L-arginine pool is also used by arginases (Arg), which catalyze the hydrolysis of L-arginine to ornithine and urea, and exists in two isoforms. *ArgI* participates in the urea cycle, and is expressed at high levels in the liver (Vericelli 2003). *ArgII* is highly expressed in the prostate and kidney, and thought to be involved in the synthesis of proline and polyamines, which control cell proliferation and collagen production (Vericelli 2003). Hence, the NO production is affected by the balance between NOS- and Arg-mediated metabolic ways (Fig.24).

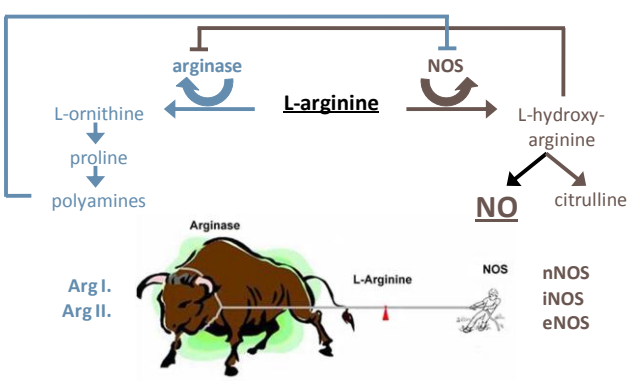


Figure 24. L-arginine metabolism. *(Israel and Peek, 2006; Vercelli, 2003)*

In the next step we investigated the expression of the the above-mentioned enzymes –except nNOS. eNOS was not detectable neither in resting, nor in AC-engulfing macrophages, suggesting that iNOS is responsible for the AC-associated NO production. In line with the higher NO production, the expression of iNOS was significantly higher in $A_{2A}R^{-/-}$ macrophages than in their wild type counterparts (Fig.25A). Exposure to ACs did not alter the levels of Arg I, but induced a downregulation in iNOS and an upregulation in the Arg II, favoring the utilization of arginine in the production of polyamines. However, both the upregulation of Arg II and the downregulation of iNOS by ACs were delayed in $A_{2A}R^{-/-}$ macrophages (Fig.25B). All these data indicate that exposure to ACs induces a modification in the arginine metabolism that favors polyamine production. But this shift is delayed in $A_{2A}R$ null macrophages.

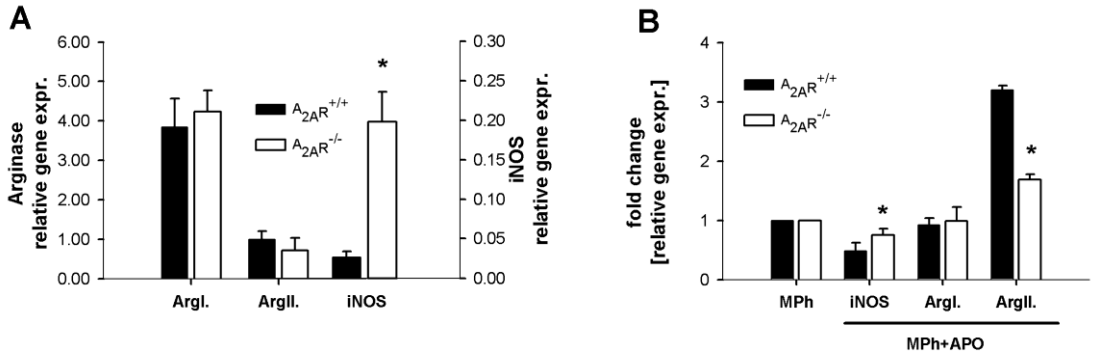


Figure 25. Loss of the adenosine $A_{2A}R$ induces changes in the mRNA expression of enzymes in arginine metabolism favoring NO production in macrophages exposed to apoptotic cells.

*(A) mRNA expression of arginase I (ArgI.) and II (ArgII.), and iNOS in resting wild type and $A_{2A}R^{-/-}$ macrophages (MPh). Expression values are represented as mean amount of target mRNA normalized to the expression of cyclophilin. (B) Changes in the mRNA expression of arginase I and II and iNOS in wild type and $A_{2A}R^{-/-}$ macrophages following exposure to apoptotic cells (APO). mRNA levels are expressed as fold changes as compared to the resting macrophages. Results are expressed as mean ± S.D. of five independent experiments (*p<0.05).*

Since the $A_{2A}R$-induced adenylate cyclase pathway suppresses NO production, we tested whether influencing the adenylate cyclase pathway alters arginase II or iNOS expression As shown in Fig.26A, inhibition of PKA by Rp-cAMP triethylamine in wild type macrophages enhances the expression of iNOS, while the addition of forskolin to $A_{2A}R^{-/-}$ macrophages inhibits it (Fig.26B). Similar manipulations altered the expression of arginase II conversely.

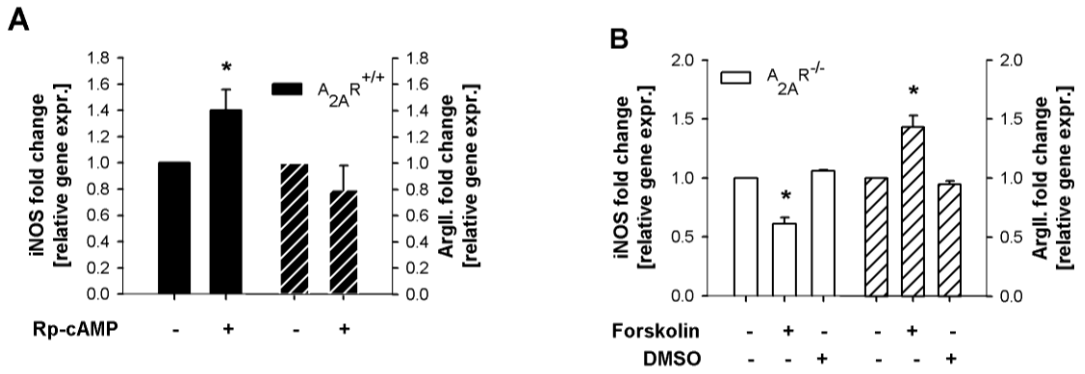


Figure 26. The adenylate cyclase pathway alters arginase II and iNOS gene expression.

*(A) Inhibition of protein kinase A in wild-type macrophages enhances, while (B) activation of adenylate cyclase in $A_{2A}R$ null macrophages decreases the mRNA levels of iNOS. The expression of arginase II (lined bars) under the same conditions is adversely affected. Expression values are represented as mean amount of target mRNA normalized to the expression of cyclophilin. Results are expressed as mean ± S.D. of five independent experiments (*p<0.05).*

5. DISCUSSION

Today it is believed that inflammation is part of the non-specific immune response that occurs in reaction to harmful stimuli, such as pathogens or damaged cells (Ferrero-Miliani *et al.*, 2007) and serves a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process.

Inflammation is stimulated by chemical factors released by injured cells and initiated by cells already present in all tissues, mainly by resident macrophages. Chemical factors produced during inflammation (such as IL-8, MCP-1, MIP-2 and fractalkine) attract phagocytes. The first cells attracted to the site of infection are generally neutrophils. They are followed by monocytes, which differentiate into more tissue macrophages. Neutrophils and macrophages not only phagocytose pathogens and infected cells, but also release variety of inflammatory mediators. These include the lipid mediators (prostaglandins, leukotrienes, and PAF), pro-inflammatory cytokines (such as IL-1, IL-6, TNF-α) and chemoattractants, which trigger other parts of the immune system (Janeway *et al.*, 2001).

The inflammatory response must be actively terminated when no longer needed to prevent unnecessary "bystander" damage to tissues (Cotran*et al.*, 1998). Emerging evidence now suggests that an active, coordinated program of resolution initiates in the first few hours after an inflammatory response begins (Serhan and Savill, 2005). Resolution of inflammation occurs by different mechanisms in different tissues. Mechanisms that serve to terminate inflammation include the short half-life of inflammatory mediators, the production and release of TGF-β (Ashcroft, 1999) and IL-10 (Sato *et al.*, 1999) from macrophages, the downregulation of pro-inflammatory molecules, and the upregulation of anti-inflammatory molecules such as the IL-1ra or the soluble TNF receptor (sTNFR), and the desensitization of receptors. In the resolution phase, neutrophil recruitment breaks off and the pro-inflammatory cells die by apoptosis (Greenhalgh, 1998). Consequently, apoptotic neutrophils undergo phagocytosis by macrophages, leading to neutrophil clearance and release of anti-inflammatory and reparative cytokines such as TGF-β (Serhan and Savill, 2005).

In the present study, on one hand we investigated the potential role of TG2 as a regulatory molecule in the inflammatory responsiveness of macrophages. We found that in the absence of TG2 macrophages become more sensitive to LPS treatment and respond by enhanced pro-

inflammatory cytokine production as compared to their wild-type counterparts. Increasing evidence suggests that TGF-β released by macrophages exposed to either LPS (Nunes *et al.,* 1995) or ACs (Fadok *et al.,* 1998) play a key role in the control or termination of the pro-inflammatory response. However, to act so macrophages have to be exposed prior or for a longer time period to the endogenous or r TGF-β. As a result, though we confirmed previous suggestions that TG2 is required for TGF-β activation by murine macrophages (Nunes *et al.,* 1995), testing by addition of neutralizing anti-TGF-β antibodies, lack of active TGF-β production did not significantly affect the LPS-induced pro-inflammatory cytokine production of TG2 null macrophages in short term cultures.

Instead, we found that loss of TG2 altered the $\alpha_v\beta_3$ integrin signaling in macrophages leading to an enhanced basal Src tyrosine kinase activity. The cross-linking activity of TG2 was not required for proper $\alpha_v\beta_3$ integrin signaling and LPS-induced cytokine production, but TG2 had to be expressed on the cell surface and bind guanine nucleotides. These results are in agreement with previous findings, which demonstrated that TG2 modifies integrin signaling in guanine nucleotide bound form (Johnson and Terkeltaub, 2005). Though TG2 can act as a G protein in many physiological settings, in the context of regulating integrin signaling proper guanine nucleotide binding of TG2 was suggested to be required for stabilizing the protein in a conformation state that can facilitate physical interactions with other proteins, such as integrins (Johnson and Terkeltaub, 2005). Our findings seem to confirm that of others, which showed a synergism between $\alpha_v\beta_3$ integrin signaling and LPS sensitivity (Lee *et al.,* 2007).

Interestingly, though loss of TG2 sensitized macrophages to LPS, loss of TG2 prevented mice from the endotoxic shock induced by LPS (Falasca et a., 2008). The pathogenesis of the endotoxic shock, however, is very complex, and the various effects of the multifunctional protein TG2 in various tissues, such as heart, kidney or neutrophils, explain the controversy between our findings and the *in vivo* results (Falasca *et al.,* 2008). Our data, however, demonstrate that TG2 null macrophages might be more sensitive to all stimuli that lead to pro-inflammatory cytokine production via activation of the NF-κB pathway. This pro-inflammatory phenotype of TG2 null macrophages might contribute to the development of autoimmunity in these mice (Szondy *et al.,* 2003) and their increased sensitivity to develop atherosclerosis (Boisvert *et al.,* 2006; De Laurenzi and Melino, 2001).

In addition to phagocytosis and inflammatory cytokine production, activated macrophages also release chemoattractant mediators, which trigger the local accumulation of neutrophil

granulocytes. Due to their high phagocytic capacity, immigrated neutrophils greatly contribute to eradicating the inflammation, but as a short-living cell types, neutrophils die quickly by apoptosis. The resolution program of inflammation involves phagocytosis of these apoptotic neutrophils by macrophages, leading to neutrophil clearance and release of anti-inflammatory and reparative cytokines such as TGF-β and IL-10 (Voll *et al.*, 1997, Fadok *et al.*, 1998). But not only the TGF-β and IL-10 are the only and obligate anti-inflammatory mediator produced by macrophages. As an endogenously produced soluble anti-inflammatory molecule, adenosine's participation was also raised in the anti-inflammatory effect of ACs.

In the other part of our work, we have shown that macrophages engulfing ACs produce ADO at levels that can trigger A_{2A}Rs and, at the same time, elevate the expression of the receptor itself. Thus, ADO can act in an autocrine manner during phagocytosis. Loss of A_{2A}Rs did not affect the rate of phagocytosis. This was a surprise for us, as increases in cAMP levels were reported to inhibit engulfment of ACs (Rossi *et al.,* 1998). However, when exposed to ACs, $A_{2A}R^{-/-}$ macrophages notably produced increased amounts of MIP-2 and KC acting as chemoattractants for various cell types, especially for neutrophils. We could confirm these data using a specific A_{2A}R agonist and an A_{2A}R antagonist indicating that lack of actual A_{2A}R signaling rather than altered macrophage differentiation in the absence of A_{2A}R explains the phenomenon. These data suggest that during engulfment of ACs, especially when macrophages participate in the resolution of inflammation, where they clear large numbers of apoptotic neutrophils (Savill *et al.,* 2002), A_{2A}Rs might participate in the negative feedback control of neutrophil transmigration to the inflammation site. There is clear evidence that both the function and expression of A_{2A}R is regulated by inflammatory cytokines (e.g. TNF-α, IL-1), which modify its desensitization by influencing the intracellular binding of GPCR kinases (GRKs) and arrestins (Nguyen *et al.*, 2003; Khoa *et al.*, 2006). Therefore, the role of ADO mediating the inhibitory effect of ACs might be more significant under inflammatory conditions, than it was observed in our *in vitro* model, which lacked inflammation. In support of this hypothesis, enhanced production of MIP-2 and KC by $A_{2A}R^{-/-}$ macrophages engulfing ACs was shown in an *in vivo* peritonitis model, and this was accompanied by MIP-2- and KC-dependent neutrophil migration which was not seen in wild-type mice. Despite their large numbers relative to tissue macrophages, neutrophil disappearance in resolving inflammation is often dramatic, such that detection of morphologically dead neutrophils is relatively infrequent in tissues during resolution (Bratton and Henson, 2011). Under *in vivo* conditions ADO acting via A_{2A}Rs of neutrophils intensifies the anti-inflammatory responses of immune sys-

tem. According to Sipka, 2011, ADO acting via the $A_{2A}R$/phospolipase A2 signaling pathway seems to be involved in the regulation of inflammatory precursor arachidonic acid, and in the production of lipid mediators (leukotrienes, prostaglandins and eicosanoids) (Sipka, 2011).

In our further experiments, MIP-2 production by $A_{2A}R^{-/-}$ macrophages was studied in details. Though previous studies have shown that AC-induced IL-10 production in macrophages can negatively regulate the production of pro-inflammatory cytokines (Voll *et al.*, 1997), and $A_{2A}Rs$ were reported in certain inflammatory contexts to promote IL-10 formation (Montesinos *et al.*, 2006), we found no detectable IL-10 production in our experimental system. Indeed, at submillimolar concentrations, adenosine enhances the production of IL-10 by monocytes. High extracellular levels of adenosine, like those leading to enhanced IL-10 secretion, are unlikely to occur during infection or inflammation where adenosine reaches the submicromolar concentration range (Le Moine *et al.*, 1996). Instead, we found that MIP-2 synthesis was partially related to an enhanced NO production by $A_{2A}R^{-/-}$ macrophages engulfing ACs that regulated MIP-2 production on transcriptional level.

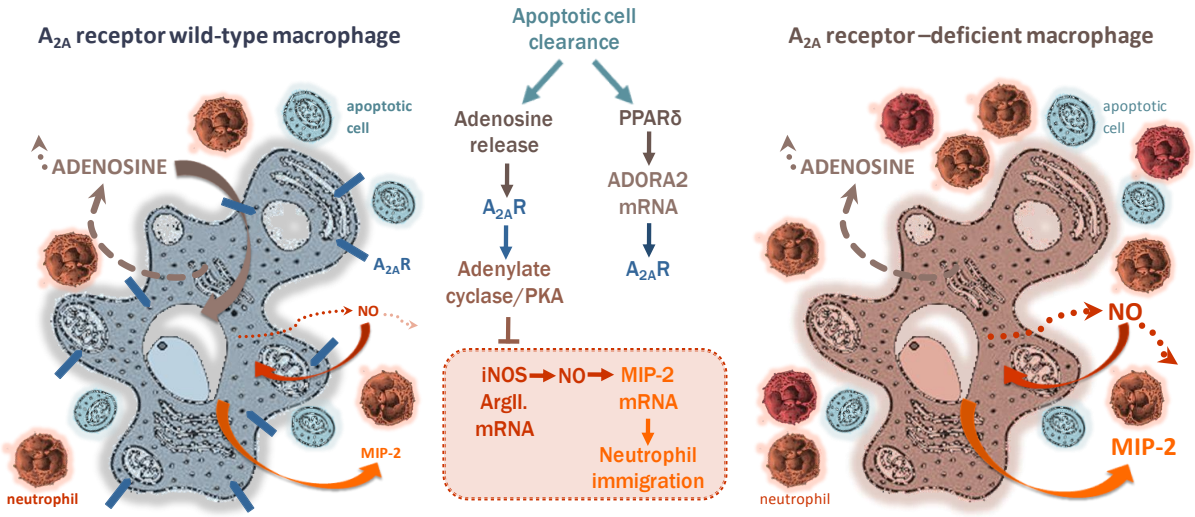


Figure 37. The role of adenosine/$A_{2A}R$ system in the prevention against overshot MIP-2 production and neutrophil immigration

Enhanced NO production of $A_{2A}R$ null macrophages as compared to the wild types seems to be related to higher levels of iNOS, which produces NO, and lower levels of arginase II, which normally degrades arginine, the substrate of NO synthesis, prior and following AC exposure. Although, iNOS expression is regulated mainly at the transcription level by the activation of several transcription factors that bind to the promoter region of the iNOS gene, such as

NF-κB, AP-1, STAT1, IRF-1 and nuclear factor-interleukin 6 (NF-IL6) (Bhatt *et al.*, 2010). However, mRNA levels alone might not reflect the real activities or activity ratio of these enzymes, as just iNOS activity alone was shown to be regulated by various signals on transcriptional, mRNA, translational and posttranslational levels (Boutard *et al.*, 1995; Bredt *et al.*, 1992). In accordance with the existence of these additional regulation levels, we observed that though iNOS is already expressed on mRNA level by resting macrophages, NO production is seen only following the AC uptake indicating that ACs must induce an early signaling pathway that triggers NO production. Our attempts to identify this signaling pathway have failed so far. Since both wild type and A_{2A}R null cells produce NO following engulfment, our current hypothesis is that A_{2A}Rs might not affect this pathway. Instead the inverse levels of iNOS and the arginase II affected by the A_{2A}Rs will determine the magnitude of the NO response. In support of our hypothesis, alterations in the arginine metabolism (favoring the arginase pathway leading to polyamine synthesis and inhibiting the synthesis of NO) following engulfment of ACs have already been reported (Freire-de-Lima *et al.*, 2000).

Interestingly, both TGF-β released by macrophages engulfing ACs (Boutard *et al.*, 1995; Vodovotz *et al.*, 1993) and compounds known to activate protein kinase A (Corraliza *et al.*, 1997; Galea and Feinstein, 1999) were shown to increase arginase activity and decrease NO production in macrophages indicating that both TGF-β and A_{2A}Rs, that activate protein kinase A, might mediate or support the effect of ACs on the arginine metabolism in macrophages engulfing ACs. The role of TGF-β was proven previously (Freire-de-Lima *et al.*, 2000), while our data indicate the additional involvement of A_{2A}Rs. All together, our data demonstrate for the first time that besides TGF-β and IL-10 (Voll *et al.*, 1997; Fadok *et al.*, 1998) ADO also participates in the negative regulation of pro-inflammatory cytokine production of macrophages engulfing ACs. In this context, ADO uses the A_{2A}R pathway and inhibits primarily neutrophil chemoattractant formation and the consequent neutrophil immigration.

Overall, studies on the anti-inflammatory effect of ADO are still the subjects of interest. Besides basic research, pharmacological and clinical studies also target the role and application of ADO analogues and A_{2A}R agonist/antagonist in various cardiovascular, neurological and chronic inflammatory diseases (Lopes et al., 2011; Szentmiklósi et al., 2011; Trevethick et al., 2008; Ohta and Sitkovsky, 2009). Our results probably give new perspectives in the therapical use of ADO/A2AR-targeted drugs in diseases connected to the disturbed clearance of apoptotic cells (e.g. SLE, atherosclerosis) (Chekeni and Ravichandran, 2011).

6. SUMMARY

Multicellular organisms respond to bacterial and fungal infection with the complex biological program of inflammation. In the initial phase of inflammation, macrophages and neutrophil granulocytes migrate to the site of infection, where they not only phagocytose pathogens, but also recruit further immune cells and induce systemic inflammatory response by the production and release of pro-inflammatory cytokines. In the resolution phase of inflammation, macrophages remove neutrohils, which died during their action. The uptake of these apoptotic cells induces phenotype shift: macrophages pass into deactivated phase, in which they release anti-inflammatory cytokine TGF-β and IL-10 instead of pro-inflammatory mediators.

In the present study we reported, that the lack of TG2 affects the inflammatory answer of macrophages, as TG2 null macrophages respond to LPS treatment by elevated IL-6 and TNF-α production. Though TGF-β has been proposed to act as a negative feed back regulator of pro-inflammatory cytokine production in LPS-stimulated macrophages, this phenomenon is not related to the lack of active TGF-β production. Instead, in the absence of TG2 integrin β3 maintains an elevated basal Src family kinase activity in macrophages, which leads to enhanced phosphorylation and degradation of the IκBα. Low basal levels of IκBα explain the enhanced sensitivity of TG2 null macrophages to signals that regulate NF-κB-mediated pro-inflammatory cytokine production. Our data suggest that TG2 null macrophages bear a proinflammatory phenotype, which might contribute to the enhanced susceptibility of these mice to develop autoimmunity and atherosclerosis.

In addition to TGF-β, macrophages engulfing apoptotic cells release adenosine in sufficient amount to trigger A2ARs, and simultaneously increase the expression of A2ARs, as a result of possible activation of LXR and PPARδ. In macrophages engulfing apoptotic cells, stimulation of A2ARs suppresses the NO-dependent formation of neutrophil migration factors, such as MIP-2, using the adenylate cyclase/PKA pathway. As a result, loss of A2ARs results in elevated chemoattractant secretion. This was evident as pronounced neutrophil migration upon exposure of macrophages to apoptotic cells in an in vivo peritonitis model. Altogether, our data indicate that adenosine is one of the soluble mediators released by macrophages that

mediate engulfment-dependent apoptotic cell suppression of inflammation, and TG2 is anti-inflammatory by both promoting active TGF-β formation and regulating integrin signaling.

7. KEYWORDS

tissue transglutaminase; integrin $\alpha_v\beta_3$; Src; inflammation; apoptotic cell clearance; adenosine; A_{2A} receptor; NO; MIP-2;

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10. PUBLICATIONS, CONFERENCES

IN EXTENSO PUBLICATIONS RELATED TO THE THESIS:

Sarang Z, Köröskényi K, Pallai A, Duró E, Melino G, Griffin M, Fésüs L, Szondy Z. **Transglutaminase 2 null macrophages respond to lipopolysaccharide stimulation by elevated proinflammatory cytokine production due to an enhanced $\alpha_v\beta_3$ integrin-induced Src tyrosine kinase signaling.** *Immunol Lett. 2011 Jul;138(1):71-8. IF:*

Köröskényi K, Duró E, Pallai A, Sarang Z, Kloor D, Ucker DS, Beceiro S, Castrillo A, Chawla A, Ledent CA, Fésüs L, Szondy Z; **Involvement of Adenosine A_{2A} Receptors in Engulfment-Dependent Apoptotic Cell Suppression of Inflammation.** *J Immunol. 2011 Jun 15;186(12):7144-55. IF:*

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Sarang Z, Tóth B, Balajthy Z, Köröskényi K, Garabuczi E, Fésüs L, Szondy Z **Some lessons from the tissue transglutaminase knockout mouse.** *Amino Acids. 2009 Apr;36(4):625-31. IF:*

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ORAL PRESENTATIONS:

Köröskényi K. **Inhibition of proinflammatory cytokine production of macrophages by apoptotic cells.** (in English) 1st Molecular Cell and Immune Biology (MCIB) Winter School, Krompachy, Slovakia January 8-11, 2008

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Köröskényi K. **Adenosine is a soluble mediator to regulate part of the anti-inflammatory responses induced in macrophages by apoptotic cells.** (in English) 3rd Molecular Cell and Immune Biology (MCIB) Winter School, Mariazell, Austria January 7-10, 2010

Köröskényi K. **Involvement of adenosine A_{2A} receptors in engulfment-dependent apoptotic cell suppression of inflammation.** (in English) 4th Molecular Cell and Immune Biology (MCIB) Winter School, Galyatető, Hungary, January 11-14, 2011

Köröskényi K. **Adenosine acting via A_{2A} receptor takes a part in the anti-inflammatory effect of apoptotic cell uptake and in this way in the termination of inflammatory response.** (in Hungarian) Annual meeting of the Hungarian Immunological Society 2010, Harkány, Hungary; October 29.−November 10, 2010

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Tóth B, Sarang Z, Köröskényi K, Garabuczi E, Aeschlimann D, Vereb G, Fésüs L, Szondy Z: **Roles of tissue transglutaminase in the phagocytosis of apoptotic cells.** 5th Euroconference on Apoptosis, Portoroz, Slovenia; October 26-31, 2007,

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Köröskényi K, Sarang Z, Fésüs L, Szondy Z: **Studies on anti-inflammatory effect of apoptotic cells in wild type and tissue transglutaminase (TG2) deficient macrophages.** (in Hungarian) Annual meeting of the Hungarian Biochemical Society 2006, Pécs, Hungary; August 30−September 02, 2006

Köröskényi K, Sarang Zs, Fésüs L, Szondy Zs: **The regulatory role of tissue transglutaminase (TG2) in the pro-inflammatory cytokine production of macrophages.** (in Hungarian) Annual meeting of the Hungarian Biochemical Society 2007, Debrecen, Hungary; August 26−29, 2007

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Köröskényi K, Sándor K, Pallai A, Duró E, Sarang Z, Fésüs L, Szondy Z **Involvement of Adenosine A_{2A} Receptors in Apoptotic Cell Induced Suppression of Inflammation.** (in English) Gordon Research Conference on Apoptotic Cell Recognition & Clearance, Lewiston, USA; July 17-22, 2011